TARGETED INHIBITION OF CONSTITUTIVE AND INTERLEUKIN-6-INDUCIBLE STAT3 SIGNALING CASCADE FOR THE PREVENTION AND TREATMENT OF HEAD AND NECK SQUAMOUS CELL CARCINOMA

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

_____

Li Feng
March 2015

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Li, F., M. K. Shanmugam, L. X. Chen, S. Chatterjee, J. Basha, A. P. Kumar, T. K. Kundu and G. Sethi (2013). "Garcinol, a Polyisoprenylated Benzophenone Modulates Multiple Proinflammatory Signaling Cascades Leading to the Suppression of Growth and Survival of Head and Neck Carcinoma." Cancer Prevention Research 6(8): 843-854. (Impact factor: 5.27)

Li, F. and G. Sethi (2010). "Targeting transcription factor NF-kappa B to overcome chemoresistance and radioresistance in cancer therapy." Biochimica Et Biophysica Acta-Reviews on Cancer 1805(2): 167-180 (Impact factor: 7.58)

Li, F., P. Rajendran and G. Sethi (2010). "Thymoquinone inhibits proliferation, induces apoptosis and chemosensitizes human multiple myeloma cells through suppression of signal transducer and activator of transcription 3 activation pathway." Br J Pharmacol 161(3): 541-554. (Impact factor: 4.99)

Li, F., P. P. Fernandez, P. Rajendran, K. M. Hui and G. Sethi (2010). "Diosgenin, a steroidal saponin, inhibits STAT3 signaling pathway leading to suppression of proliferation and chemosensitization of human hepatocellular carcinoma cells." Cancer Lett 292(2): 197-207. (Impact factor: 5.02)

Poster Presentations in Conferences

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List of Abbreviations

4-NQO	4-nitroquino-line-1 oxide
BAFF	B-cell activating factor
Bcl-xL	B-cell lymphoma-extra large
BSA	Bovine serum albumin
c-FLIP	Cellular FLICE inhibitory protein
cIAP	Cellular inhibitors of apoptosis
$cPLA_2$	Cytosolic phospholipase A_2
CBP	Cyclic AMP response element-binding protein
CDK	Cyclin-dependent kinase
CIS	Carcinoma in situ
COX-2	Cyclooxygenase-2
CT	Computed tomography
CXCR4	CXC chemokine receptor 4
DAPI	4’,6-diamidino-2-phenylindole
DMBA	7, 12-dimethylbenz[a]anthracene
DMEM	Dulbecco’s modified Eagle medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DR	Death receptor
ECM	Extracellular matrix
EdU	5-ethynyl-2’-deoxyuridine
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay

EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FADD	FAS-associated death domain
FAK	Focal-adhesion kinase
FAT	Factor acetyltransferase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FLICE	FADD-like IL-1β-converting enzyme
FOXP3	Forkhead box P3
GADD153	DNA damage-inducible gene 153
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GSK-3β	Glycogen synthase kinase-3β
HAT	Histone acetyltransferase
HB-EGF	Heparin-binding EGF-like growth factor
HDAC	Histone deacetylase
HER1	Human epidermal growth factor receptor 1
HIF1	Hypoxia-inducible factor 1
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
HPV	Human papillomavirus
i.p.	Intraperitoneal
iNOS	Inducible nitric oxide synthase
ICAM-1	Intercellular adhesion molecule 1

IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IκBα	Inhibitor of kappaB α
IKK	IκB kinase
IL	Interleukin
IR	Ionizing radiation
JAK	Janus kinase
LOH	Loss of heterozygocity
LOX	Lipoxygenase
LPS	Lipopolysaccharide
miRNA	MicroRNA
mPGES-1	Microsomal prostaglandin E synthase-1
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEKK	Mitogen-activated protein kinase/ERK kinase kinase
MET	Mesenchymal-epithelial transition
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MTT	Thiazoyl blue tetrazolium bromide
nAChR	Nicotinic acetylcholine receptor
NEAA	Non-essential amino acid
NF-κB	Nuclear factor kappa B
NHEJ	Non-homologous end joining

NIK	NF-κB-inducing kinase
NSCLC	Non-small-cell lung carcinoma
PARP	Poly (ADP-ribose) polymerase
PCAF	p300/CBP associated factor
PDK1	Phosphoinositide-dependent protein kinase-1
PG	Prostaglandin
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIAS3	Protein inhibitors of activated STAT3
PKC	Protein kinase C
PMSF	Phenylmethylsulfonylfluoride
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
PTP	Protein tyrosine phosphatase
RB	Retinoblastoma
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
SCCOT	Squamous cell carcinoma of the oral tongue
SCID	Severe combined immunedeficient
SDF-1	Stromal cell-derived factor-1
SDS	Sodium dodecyl sulfate
SHP	Src homology 2 domain-containing PTP
SOCS	Suppressor of cytokine signaling
STAT	Signal transducers and activators of transcription

TAK1	TGF beta activated kinase 1
TCM	Traditional Chinese medicine
TCR	T-cell receptor
TGF-α	Transforming growth factor alpha
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

Summary

Head and neck squamous cell carcinoma (HNSCC) refers to a group of biologically similar cancers which start in the upper aerodigestive tract, that 90% of them are squamous cell carcinomas originating from the mucosal lining of these regions. Platinum compounds such as cisplatin and carboplatin are frequently used as the first-line chemotherapy for the treatment of the HNSCC. However, the therapeutic efficacies of these agents are often quite limited due to poor prognosis and development of resistance; moreover, high doses of platinating agents which are required for significant anti-tumor effect frequently lead to severe side effects that compromise the quality of life of the patients. Hence, novel pharmacological agents that are safe and can overcome chemoresistance are urgently required. Natural products offer rich and powerful sources of therapeutic leads for the drug development that could provide effective alternative strategies. Garcinol, a poly-isoprenylated benzophenone, is one of the major active compounds extracted from the dried rind of the fruit of *Garcinia indica* tree that has been reported to exhibit significant anti-neoplastic and chemopreventive roles in various tumor cell lines and *in vivo* tumor models in increasing number of studies in recent years. However, the molecular mechanism(s) through which garcinol elicits its anti-tumor activity, specifically in HNSCC, is unknown till date. Thus, in the present study, we investigated the potential effects of garcinol on human HNSCC, and analyzed whether garcinol could sensitize HNSCC to cisplatin *in vitro* and in a xenograft mouse model.

Firstly, we found that garcinol could exhibit diverse anti-cancer effects as it inhibited the proliferation of HNSCC cell lines, induced apoptosis, and abrogated HNSCC cell migration and invasion. When tested together with the conventional chemotherapeutic agent, garcinol could enhance the cytotoxic and apoptotic effects of cisplatin in a synergistic manner in HNSCC cells. We observed that garcinol suppressed the activation of various upstream kinases of STAT3 signaling cascade, thereby inhibited the constitutive and IL-6-induced STAT3 phosphorylation, nuclear translocation and DNA binding activity. Garcinol also exhibited an inhibitory effect on constitutive NF-κB activation, mediated through the suppression of TAK1 and IKK activation in HNSCC cells. We further noticed that the chemotherapeutic drug cisplatin was able to induce NF-κB activation, this cisplatin-induced NF-κB activation could also be significantly abrogated upon ga rcinol treatment. Furthermore, garcinol down-modulated the expression of various STAT3 and NF-κB-regulated oncogenic gene products critical for HNSCC cell growth, survival, angiogenesis, metastasis and chemoresistance. Finally, whether garcinol can sensitize the HNSCC to cisplatin treatment was investigated in HNSCC xenograft mouse model. Our i*n vivo* study showed that administration of garcinol alone significantly suppressed the growth of human HNSCC xenograft tumors in athymic nu/nu mice, and the combination treatment of garcinol and cisplatin exhibited greater anti-tumor effect. Analysis of tumor tissues further verified the inhibitory effects of garcinol on STAT3 and p65 activation in tumor samples from garcinol treated mice, and this

is correlated with the suppression of various markers of proliferation, survival, angiogenesis and invasion.

Taken together, the present findings clearly demonstrate that anti-cancer activities of garcinol in human HNSCC cell lines and in xenograft mouse model, are mediated through the modulation of multiple pro-inflammatory signaling cascades, and thereby highlight the great potential of garcinol as an effective anti-cancer agent against HNSCC and other malignancies.

1 INTRODUCTION

1.1 Cancer

Cancer, known clinically as a malignant neoplasm, is a group of diseases caused by uncontrolled growth of cells (1). Cancer is a major public health problem worldwide, with 12.7 million cases and causes 7.6 million cancer deaths estimated by the International Agency for Research on Cancer (IARC) in 2008 (2). The burden of cancer is projected to rise continuously due to global population growth and aging, and increasingly adoption of cancer-causing lifestyle (3). In the Singapore Cancer Registry Interim Annual Registry Report, it is reported cancer incidents have increased year on year from the year of 2008 to 2012 in Singapore. A total of 56,316 cases were diagnosed during this period.

Normal cells evolve progressively to neoplastic state through the assemblage of genetic mutations and epigenetic alterations that confer them a selective survival advantage (4). Hanahan and Weinberg have described the key hallmark capabilities acquired by the cancer cells during the multistep process of human tumor pathogenesis. The first proposed six hall marks of cancer include self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue

invasion and metastasis (5). Recently, enabling characteristics (genomic instability and mutation, and tumor promoting inflammation) and emerging hallmarks (reprogramming energy metabolism, and evading immune destruction) have been added to the list (4). In addition, the interactions between the cancer cells and tumor microenvironment contribute to the entire process of cancer etiology, progression and metastasis (6).

1.2 Head and Neck Squamous Cell Carcinoma (HNSCC)

Head and neck cancer is a complex disease which comprises a broad range of biologically similar epithelial malignancies start in the upper aerodigestive tract, including the lip, nasal cavity, oral cavity, paranasal sinuses, pharynx, and larynx (7, 8), and accounts for approximately 650,000 new cases and 350,000 cancer-related deaths worldwide annually (6.0% of cases and 5.2% deaths) (9). Almost all of the head and neck cancers are squamous cell carcinomas originating from the mucosal lining of these regions (10).

1.2.1 Epidemiology

Use of tobacco, alcohol consumption, and human papillomavirus (HPV) infection are the major risk factors for head and neck cancer, with tobacco and alcohol having interactive and synergistic effects (11, 12). Subsets of HNSCC, especially oropharyngeal cancer are identified to be attributed to high-risk type HPV infection (13, 14). The distributions of HNSCC subtypes are variant, for instance, the cancers of the lip and oral cavity is mostly found in Melanesia and southcentral Asia, while nasopharyngeal cancer is more prevalent in southeast Asia region (3), such variations are strongly associated to the relative distributions of major risk factors (15). According to the Singapore Cancer Registry Interim Annual Registry Report, nasopharyeal cancer ranks as the eighth most frequent cancers in males in Singapore at the year of 2008-2012 (16). As one of the commonest cancers in Singapore, more than 800 new cases of HNSCC are diagnosed per year (17).

1.2.2 Diagnosis and staging

Early recognition of signs and symptoms is important for prompt diagnosis; however the symptoms of early stage HNSCC are usually vague. The

signs/symptoms vary with the anatomic location of primary tumor and the stage of the cancer (7). For example, common symptoms for early nasopharyngeal cancer include nasal obstruction, epistaxis, and serous otitis media, and patients with advanced tumor present with cranial neuropathies and posterior cervical lymphadenopathy (7). Whereas, cancers of the oropharynx, hypopharynx, and supraglottic larynx only produce symptoms such as persistent unilateral sore throat and otalgia in later stages (7). As a biofluid in close contact with HNSCC lesion, saliva has been studied for developing biomarkers to detect early cancers and minimal residual disease in a non-invasive manner (18). Several strategies have been designed to identify biomarkers such as HPV status, promoter hypermethylation profiles, telomerase activity, and differential gene expression profiles (18). Indeed, aberrant promoter methylation patterns of cancer-related genes in saliva and serum from HNSCC patients have been found to match the methylation profiles of primary tumors (19, 20). All these efforts have revealed the feasibility of saliva-based strategies for the early diagnosis of HNSCC.

Accurate staging depends on multidisciplinary consultations usually involve complete head and neck examination (mirror and fiberoptic examination) coupled with other appropriate studies (biopsy, chest imaging, computed tomography (CT)

and/or magnetic resonance imaging (MRI) scan, tumor HPV test for oropharyngeal cancer and PET-CT for stage III/IV disease) as required (21). The TNM (primary tumor (T), regional lymph node (N) and involvement distant metastasis (M)) staging system developed by the American Joint Committee on Cancer (AJCC) is widely used; it integrates all clinically available information and guides the therapeutic decision making (22).

1.2.3 Field cancerization

The term “field cancerization” was first proposed by Slaughter to describe the occurrence of many precancerous and cancerous lesions at multi-focal "field" along the upper aerodigestive tract owing to the prolonged exposure to environmental carcinogens such as tobacco and alcohol, as well as infection with high-risk oncogenic HPV types (8, 23, 24). There are frequent observations of abnormal tissues surrounding the tumors, and high rate of lateral occurrence of local recurrences and second primary tumors. The recent advances of techniques in molecular and genetic analysis revealed that the areas of histopathological abnormality surrounding malignant lesions and the primary tumor share genetic alterations as a indication of common clonal origin (25, 26). Different genetic

alterations take place in the subsequent development of various subclones, results in a variety of histopathologically diverse regions in a local anatomical area (27, 28).

1.2.4 Multistep carcinogenesis in HNSCC

The multistep progression of HNSCC from premalignant lesion to invasive cancer type is characterized by the accumulation of genetic and epigenetic alterations associated with the morphological changes in the squamous epithelium (27-29). HNSCC is initiated and grows by the process of clonal evolution driven by multiple mutations in the mucosa cells and subsequently progress to cytologically recognizable premalignant foci of dysplasia, and eventually to carcinoma in situ (CIS) or cancer (27, 29). Benign squamous hyperplasia which usually appears similar to normal mucosa already harbors early genetic changes, often with loss of heterozygocity (LOH) at chromosomal loci 9p and inactivation of *p16*. Further loss of chromosomes 3p and 17p along with the *p53* mutation occurs in dysplasia, reflecting early tumorigenesis; whereas additional losses on 4q, 8p and 11q are typically present in carcinoma in situ or invasive squamous cell carcinomas (27, 28).

1.2.5 Genetic and epigenetic alterations in HNSCC

1.2.5.1 $p16^{INK4A}$-cyclin D1-CDK- Retinoblastoma (RB) axis

The tumor suppressor gene $p16^{INK4A}$ is the dominantly disrupted component of RB pathway frequently encountered in HNSCC. The inhibitor of cyclin-dependent kinases (CDK) 4 and 6, p16 is commonly inactivated by gene mutation, homozygous deletion, and promoter hypermethylation in the early progression of HNSCC (30-32). Indeed, chromosomal loss at 9p21 where $p16^{INK4A}$ resides occurs in up to 80% of HNSCC patients. The loss of functional p16 confers the cancer cell the ability to escape from the replicative stress-induced senescence (33). Overexpression of cyclin D1 and amplification of 11q13 (where *CCND1* is located), are found frequently in HNSCC and is usually associated with more aggressive disease (34-36). Rb mutations are rare in HNSCC, but loss of Rb have been observed in oral cancer with lower frequency (37-39), suggesting that further alterations in p16-Rb pathway would provide limited growth advantage in the presence of p16 inactivation, or Rb function maybe perturbed by E7 viral protein binding in HPV-positive cancers (40).

1.2.5.2 TP53

Inactivation of functional tumor suppressor TP53 is one of the most frequent genetic alterations in the early progression of HNSCC. Somatic mutation of TP53 and loss of heterozygocity of 17p13 where the TP53 gene resides is presented in more than 50% of the HNSCC (41-43). In HPV-positive tumors, loss of TP53 function is mediated by the destruction of p53 protein induced by E6 viral oncoprotein (44). Abrogation of p53 may lead to the increase of unchecked genetic alterations due to the absence of proper cellular response to DNA damage which may result in further tumor progression (45).

1.2.5.3 EGFR pathway

Epidermal growth factor receptor (EGFR; ErbB-1; HER1 in humans) is a member of ErbB/HER family receptor tyrosine kinase that mediates the cellular effects in response to binding of ligands (46). EGFR can be activated by several natural ligands, including epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, epiregulin, betacellulin, and heparin-binding EGF-like growth factor (HB-EGF) (47). Upon ligand binding on the extracellular domain, EGFRs form homo- or hetero-dimers and stimulate the intrinsic

intracellular tyrosine kinase activities which transduce signals to regulate comprehensive signaling cascades involved in a wide range of cellular activities such as gene expression, cellular proliferation, differentiation and cell migration (46, 48). EGFRs are expressed differentially in tissues of epithelial, mesenchymal and neuronal origin, where they are required for various biological/developmental processes; it is critical in modulating specific aspects of vertebrate embryogenesis, and important in mammary gland development by promoting ductal growth (49, 50).

Soon after its discovery in 1978, EGFR was linked directly to human tumors (51). The deregulation of EGFR pathway can be achieved through different mechanisms in multiple cancers, such as gene mutation, overexpression or amplification of a component of the pathway. Overexpression of EGFR ligands, in particular TGF-α and EGF, would stimulate the EGFR cascade through autocrine or paracrine loop, which are often associated with poor survival and prognosis in broad range of cancers such as lung, colon, prostate and pancreatic carcinomas (47, 52, 53). Overexpression of EGFR by gene amplification or increased translation has been reported in diverse tumors like carcinomas of lung, breast and glioma usually coupled with the elevated level of its cognate ligands

(54, 55). Genetic mutations of EGFR have been identified by several studies, with epidermal growth factor receptor variant III (EGFRvIII) being the most prevalent one found in brain tumor, which result in the constitutive activation of the receptor (56). Carcinomas of the lung, breast and ovary have been shown to express the EGFRvIII variant, which implies broader implications to human cancers (57). Studies have demonstrated that over-expression of normal EGFR is able to induce cellular transformation in the presence of appropriate levels of ligands, and co-expression of EGFR and other ErbB family receptors is necessary to induce full transformation *in vitro and in vivo* (58).

EGFR plays a very importance role in the pathogenesis of HNSCC. More than 90% of the HNSCC express increased level of EGFR mRNA and overexpression of EGFR protein has been observed in almost 50% of the head and neck tumors (59, 60), and EGFR overexpression in HNSCC has been associated with poor survival rates, decreased radiation sensitivity and higher risk of tumor recurrence (55, 61, 62). Autocrine and paracrine stimulation of EGFR by the ligands and mutational activation of EGFR gene have been also revealed as important mechanisms involved in the deregulation of EGFR in subsets of HNSCC (63, 64). EGFR acts as a central signal transducer of multiple important

signaling pathways including Ras-Raf-mitogen activated protein kinase (MAPK) cascade, phosphatidylinositol-3-kinase (PI3K)-phosphatase and tensin homolog (PTEN)-AKT axis, signal transducers and activators of transcription (STAT) pathway and phospholipase Cγ signaling which contribute to tumor growth, survival, angiogenesis, invasion and metastasis (65).

1.2.5.4 STAT3 signaling cascade in HNSCC

A network of cytokine and growth factors is essential in regulating the distinct biological processes of embryogenesis, immunity, inflammation, and hematopoiesis, many of which rely on the STAT pathway (66). The STAT family of transcription factors was first uncovered in the study of interferon (IFN) signaling by James E. Darnell (67). Seven STATs family members have been identified up to now, namely STAT 1, 2, 3, 4, 5a, 5b, and 6, which mediate the transcription of variety of genes involved in the development, immune response and growth control (68). STAT3 was first described as transcription factor that selectively binds to an enhancer element in the promoter region of acute-phase genes in interleukin-6 (IL-6) stimulated hepatocytes (69). Subsequently, it has been found that STAT3 signaling can be activated in response to various

cytokines and growth factors (70, 71). Compared to the other members of the family, STAT3 plays more diverse roles in the various biological activities; for example, STAT3 is involved in acute phase response, macrophage and B cell differentiation, migration of leukocyte, epidermal cell, and keratinocyte, and maintenance of the pluripotent state of proliferating embryonic stem cells (72, 73).

In the 1990s, the link of STAT3 signaling and oncogenesis was established with the findings that STAT3 is constitutively phosphorylated STAT3 in the v-Src transformed cells and such activation is essential for the Src-mediated oncogenesis (74, 75). Moreover, expression of constitutively activated STAT3 in fibroblast is sufficient to mediate cellular transformation, and the transformed cells can form tumor in nude mice (76). Abnormal elevated levels of STAT3 has been detected at high frequency in numerous human malignancies, including leukemia's, lymphomas, multiple myeloma, as well as solid tumors like breast cancer, prostate cancer, renal cell carcinoma and HNSCC (77). The importance of STAT3 in oncogenesis has been further evidenced by the studies demonstrating cell growth inhibition and apoptosis using STAT3 dominant negative construct (78, 79). Furthermore, STAT3 has been found to contribute to different stages of carcinogenesis. STAT3 signaling supports tumor cell survival and proliferation by

upregulating anti-apoptotic factor B-cell lymphoma-extra large (Bcl-xL), survivin, cyclin D1 and c-Myc (71). Recently, STAT3 has been shown as the direct transcriptional activator of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1 (HIF1) and matrix metalloproteinases (MMPs) (80, 81). STAT3 also plays important roles in tumor inflammation and immunity by promoting pro-oncogenic inflammatory cascades, and by opposing anti-tumor immune responses (70).

Several lines of evidence(s) have indicated that the constitutive activation of STAT3 is an early event in head and neck carcinogenesis (82-84), with tumors and adjacent mucosa from HNSCC patients exhibiting elevated STAT3 level, increased tyrosine phosphorylation and DNA binding activity compared to healthy individuals (82). Activation of STAT3 by phos phorylation in tumor is also linked to lower survival rates of HNSCC patients, thereby supporting a prognostic role of activated STAT3 (85, 86). Studies using antisense treatment and transfection of dominant-negative STAT3 constructs resulted in cell growth inhibition and apoptosis in HNSCC, provided promising evidence that constitutive STAT3 activation plays an essential role in HNSCC (82, 87). Several genes such as *Bcl-xL*, *Mcl-1*, and *survivin* which are regulated by STAT3 may

confer the survival advantage to tumors; among them, *Bcl-xL* is important in HNSCC as d ecreased expression level of *Bcl-xL* is observed in HNSCC xenografts treated with STAT3 antisense gene therapy (82). Furthermore, HNSCC cells transfected with dominant-active STAT3 mutant construct demonstrated increased proliferation both *in vitro* and *in vivo,* indicating that STAT3 activation can lead to HNSCC growth independent of growth factor stimulation (84). Given the pivotal role of activated STAT3 signaling cascade in the growth, proliferation and survival of HNSCC, molecular agents targeting STAT3 and the STAT3 signaling pathway such as duplex oligonucleotide of STAT3 (STAT3 decoy), phosphotyrosyl peptides, related peptidomimetics and small molecule compounds have been developed and found to inhibit growth of HNSCC cells both *in vitro* and *in vivo* (87-90).

1.2.5.5 NF-κB signaling cascade in HNSCC

Nuclear factor kappa B (NF-κB) was first identified as a DNA binding protein that specifically bound to the immunoglobulin κ light chain enhancer which is restricted in B cells by D avid Baltimore in 1986 (91). NF-κB is a Rel family transcription factor which consists of five members in mammalian cells,

namely, RelA (p65), and RelB, Rel (c-Rel), NF-κB1 (p50/p105) and NF-κB2 (p52/p100) (92). Both classical and alternate pathways can activate the NF-κB signaling through IκB kinase (IKK) dependent manner. In the canonical pathway, the activated β subunit of IKK (IKKβ) phosphorylates the negative regulator of NF-κB, inhibitor of kappa B-α (IκBα) protein upon the activation of IKK complex, thereafter leads to the ubiquitination and proteasome-mediated degradation of IκBα. This releases the p65/p50 heterodimer and allows the translocation of NF-κB complex to nucleus (93). Activation of non-canonical pathway involves the NF-κB-inducing kinase (NIK) mediated activation of IKKα homodimer which then activates p100/RelB by proteasomal degradation of its inhibitory C-terminal half for processing into p52/RelB heterodimer (94). Various pro-inflammatory cytokines, tumor necrosis factor (TNF), lipopolysaccharide (LPS) and other stimuli like DNA-damaging agents and viral proteins, working through the TNF receptor (TNFR), Toll-like receptor/ interleukin-1 (TLR/IL-1R) and T-cell receptor (TCR) activate the classical NF-κB pathway (93). Following the ligand receptor binding, signaling proceeds through TNFR-associated factor/receptor-interacting protein (TRAF/RIP) complexes, usually with the engagement of TGF beta activated kinase 1 (TAK1), leading to canonical signaling (95). On the other

hand, the alternative pathway is stimulated by a more restricted set of cytokines that belong to the TNF superfamily, such as B-cell activating factor (BAFF), LTβ and CD40 (96). It is well established that the canonical NF-κB pathway is essential for inflammation and innate immunity, while non-canonical pathway plays a central role in the lymphoid organ development and adaptive immunity (97). In general, NF-κB family proteins are evolutionarily conserved mediators which integrate multiple stress stimuli to regulate innate and adaptive immune responses; they act broadly to influence gene expression events that impact cell survival, differentiation, proliferation, adhesion, immunity and inflammation (98, 99).

Aberrant NF-κB activation has been implied in the pathogenesis of various human diseases such as inflammatory diseases, metabolic disorders, and cancers which are related to inflammation, oxidative stress and enhanced cell proliferation (100, 101). The first evidence implicating the oncogenic potential of NF-κB was the identification of retroviral oncoprotein v-Rel which shares a Rel transactivation domain with the mammalian homologues (102, 103). Numerous evidences have shown that constitutive activation of NF-κB is prevalent in most major human cancers mainly due to the aberrant activation of upstream signaling

molecules, or through the autocrine or paracrine activation by cytokines and growth factors, and sometimes by the genetic alteration of genes encoding NF-κB and IκB proteins (104, 105). The involvement of NF-κB has been reported in different stages of cancer development and progression. Recent studies using genetic mouse models have provided direct evidences that the IKKβ-dependent NF-κB activation is essential for initiation and promotion of inflammation-associated cancer (106, 107). NF-κB stimulates the growth of malignant cells by activating pro-inflammatory genes such as granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-6 and TNF as well as cell cycle regulators cyclin D1, CDK2, and c-Myc (108, 109). NF-κB also inhibits programmed cell death by inducing target genes including Bcl-2 family members, cellular inhibitors of apoptosis (cIAPs) and caspase-8/FADD (FAS-associated death domain)-like IL-1β-converting enzyme (FLICE) inhibitory protein (c-FLIP) (110). NF-κB promotes angiogenesis which facilitate the vascularization of tumors via directly or indirectly mediating angiogenic factor VEGF and chemokine such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) (111, 112). The expression of various adhesion molecules including intercellular adhesion molecule 1 (ICAM-1), ELAM-1, and VCAM-1,and several matrix metalloproteinases such as MMP2

and MMP9 have been found to be regulated by NF-κB, that implicated the mediating role of NF-κB in the processes of epithelial-mesenchymal transition (EMT) and metastasis (113, 114). Accumulating evidence(s) over the last few years indicate that most chemotherapeutic agents and radiation therapy induce the activation of NF-κB and its mediator genes in different type of cancers which leads to the chemo- or radio-resistance in tumor cells, and inhibition of NF-κB increases sensitivity of cancer cells to the apoptotic action of chemotherapy and to radiation exposure (115).

Many studies have documented the prevalence of constitutive activated NF-κB in HNSCC cell lines and tumor tissues specimens. For example, carcinogens in tobacco, autocrine or paracrine secretion of IL-1α, EGF and TGF-α, as well as chronic HPV infection which are linked to tumorigenesis induce NF-κB activation in HNSCC cell lines (116-120). The dysregulated NF-κB signaling modulates the expression of programs of functional genes that contributes to cell proliferation (*cyclin D1*), cell survival and therapeutic resistance (*IL-6, cIAP1, Bcl-xL, YAP1*), angiogenesis (*VEGF, IL-8*), immune and pro-inflammatory responses (cytokines, chemokines, cyclooxygenase-2 (*COX-2*) and invasion and metastasis (*MMP9, ICAM-1*) of HNSCC (121-125). Global gene profiling

analysis has demonstrated that NF-κB signaling significantly contributes to metastatic progression of HNSCC (126), and identified the activation of NF-κB as a major biomarker for high-risk HNSCC patients (127). Elevated phosphorylation level of NF-κB in patient samples is associated with poor prognosis in terms of high recurrence and poor survival (128). Different experiments have been designed to block the deregulated NF-κB activation to study the important role of NF-κB in HNSCC development and progression; such strategies include the overexpression of dominant-negative mutant of IκBα (IκBαM) which does not respond to IKK phosphorylation (129), expression of IKKα and IKKβ kinase dead mutants (130), and using pharmacological inhibitor bortezomib (131). The inhibition of NF-κB activation in HNSCC significantly suppresses the expression of NF-κB-modulated genes, such as pro-inflammatory cytokines (*IL-6 and IL-8*), and results in the induction of cell death and tumor growth inhibition (124, 132).

1.2.6 Treatment options

Current treatments of HNSCC are complicated, depending on the primary tumor sites, stages of the disease, and considerations for organ preservation, thus multidisciplinary approaches involving surgical excision, radiation therapy, and

chemotherapy are applied (133). Approximately one-third of the HNSCC patients present with highly confined stage I or II disease without regional metastases, while the other two-thirds of all patients present with advanced stage III or IV tumors with low locoregional control rates and 5-year survival rates below 50% (134, 135). Generally, patients with early-stage tumors often undergo surgical excision alone or radiation therapy alone with curative intent, which can be achieved in up to 90 percent of patients with stage I disease and more than 60 percent of those with stage II disease (8). For patients with local regionally advanced diseases (stage III or IV), combined modality therapy has been developed as the standard of care. The major strategies include concomitant administration of chemotherapy and radiotherapy (chemoradiotherapy), induction (neoadjuvant) chemotherapy followed by surgery or radiotherapy, and adjuvant chemotherapy after the patient has been rendered free of disease (7, 136, 137). However the prognosis for the patients with locally advanced HNSCC is usually poor, that majority of them will ultimately develop local recurrence or distant metastatic disease (8, 136). Cisplatin (*cis*-dichlorodiammineplatinum (II)) has been approved by Food and Drug Administration (FDA) for the treatment of HNSCC since 1970s, and still remains as the standard and first-line chemotherapy

in treating HNSCC although many other agents such as 5-fluorouracil, bleomycin, docetaxel and methotrexate, and have been extensively studied (138, 139). In recent years, modulation of the dysregulated EGFR has been successfully exploited for the treatment of HNSCC, novel agents of such purpose include monoclonal antibodies (cetuximab, panitumumab) which target the extracellular domain of EGFR, and small molecule tyrosine kinase inhibitors (TKIs) (erlotinib, gefitinib) that target the intracellular EGFR domain (139-141).

1.2.7 Drugs in the clinical trials

Several clinical trials are currently being carried out using number of pharmacological agents for HNSCC treatment. Few such trials are listed briefly in Table 1. Although cetuximab has been approved by FDA, there are still several ongoing Phase III trials run in new patient populations to broaden and diversify the base of evidence supporting the use of cetuximab (135). A fully humanized IgG2 antibody panitumumab which differs from cetuximab in both efficacy and tolerability has just entered Phase III evaluation in HNSCC (142). Anti-angiogenic therapies have been approved in treating several solid tumors including non-small-cell lung carcinoma (NSCLC), and are being assessed in the

context of HNSCC (143). The therapies include VEGF ligand-targeted monoclonal antibody bevacizumab and small molecule VEGFR targeted TKIs such as vandetanib, sunitinib and sorafenib which possess multikinase inhibiting activity. Apart from the above mentioned drugs, there are several other agents in clinical trials including but not limited to those that target the signaling cascades involved in the pathogenesis of HNSCC (Akt inhibitor Perifosine, Src inhibitor Dasatinib and Saracatinib) (144, 145), gene therapy (Gendicine) which uses recombinant adenovirus encoding human p53 tumor suppressor gene (rAd-p53) (146), and HPV peptide epitope vaccine against HPV positive HNSCC (147). Despite the availability of various pharmacological agents, unfortunately, majority of the patients relapse after initial response or develop resistance to chemotherapy. Moreover, most of the drugs lead to frequent and severe side effects such as nausea, vomiting, mucositis, infusion reaction, dermatological toxicity, neurotoxicity, and renal dysfunction that compromise the quality of life (QOL) of the patients (148-150). For the above reasons, novel agents with lower toxicity which can enhance the effects of current chemotherapeutic drugs and overcome the chemoresistance are urgently required.

1.2.8 HNSCC animal models

Animal models that resemble similar biological system to human are invaluable in the drug discovery research in understanding biochemical and disease pathways, target identification and validation, and developing new therapeutic strategies (151). The use of appropriate animal models in the study is essential since different models have their own advantages and disadvantages. The most commonly used model is the subcutaneous transplanted xenograft of HNSCC in athymic nude mice or severe combined immunedeficient (SCID). Human tumor cells can be tolerated by the compromised immune system in nude mice that enables such cross-species "xenografted" tissues (152). Thus, the subcutaneous xenografts are easy to establish and allow easy monitoring of tumor growth and end point study. However, the tumors are not locally invasive and do not metastasize when placed subcutaneously in nude mice, thus they do not present the aggressiveness of the disease which are commonly seen in human. To improve the system, orthotopic xenograft models have been introduced, and the pathology and host microenvironments of HNSCC are more closely mimicked by implanting tumor cells into the original anatomical sites. A number of studies have established orthotopic models of sublingual model of squamous cell

carcinoma of the oral tongue (SCCOT) through injection of human cell lines or human tumor specimens into the oral tongue of nude mice, which led to the cervical lymph node and pulmonary metastasis (153-155). Besides the easily accessible oral tongue, few other orthotopic models of HNSCC have been developed, including that of a thyroid carcinoma by injecting cancer cells into the thyroid gland (156), model of sinonasal malignancy with a human epidermoid tumor cell lines injected into the maxillary sinus (157), and a model of salivary cancer in the parotid glands of nude mice (158). Compared to subcutaneous xenograft, orthotopic xenograft models are more technically challenging to establish and may result in animal morbidity and even death, and also bring difficulty in continuous evaluation for poorly accessible orthotopic sites. Despite the conveniences, these xenograft models have several shortcomings. For example, lack of a competent immune response in the host precludes the study of the important interactions between the experimental therapeutic regimens and a functional host immune system. Most importantly, these systems are not able to recapitulate the multistep carcinogenesis nature and the heterogeneity of the disease in the complex and evolving tumor-host stroma interactions (159). In order to study tumor tissue in an immunocompetent environment, syngeneic

animal models are generated; the establishment of the model involves the transplantation of tumor cells which are genetically identical (syngeneic) to the recipient animal into an immunologically competent animal environment. This model has been utilized for a variety of studies, including the evaluation of novel chemotherapeutic regimens such as vitamin D analogues and lactoferrin (160, 161) and immunotherapeutic strategies using IL-12 and IL-2 (162). The main disadvantages of the syngeneic tumor models are that they are derived from homozygously inbred mice and therefore do not represent the complexity of human tumors (163). Another widely used HNSCC models *in vivo* are the chemically-induced cancer models which employ the direct application of a potent chemical carcinogen to induce HNSCC tumor formation in immunocompetent animals. Carcinogen 7, 12-dimethylbenz[a]anthracene (DMBA)-induced Syrian hamster cheek pouch model and 4-nitroquino-line-1 oxide (4-NQO)- induced oral carcinogenesis model are the two dominant models applied in multiple studies of cancer biomarkers, therapeutic modalities and strategies as well as chemopreventive agents (164, 165). To develop the chemically-induced model, exposure to carcinogens for long durations are required and the tumor formation usually takes about 20 to 28 weeks with fairly

low frequency, and also the tumors do not resemble the aggressive features of HNSCC seen in human (165). Genetically engineered mouse models especially the inducible transgenic mouse models provide have advanced the understanding of tumorigenesis in the study of HNSCC. Examples of these elegant approaches include inducible TGF-β1 transgenic mice using progesterone receptor (PR) system (166), and conditional knockout of TGF-β type II receptor (TGFβRII) and conditional "knock-in" of oncogenic K-ras (KrasG12D) in Cre/LoxP system (167, 168).

1.3 Identification of garcinol as a potential anti-cancer agent

Natural products for ancient medical uses such as t raditional Chinese medicine (TCM) and Ayurveda practiced in India are gaining recognition by the scientific and medical communities in the Western world in recent years (169, 170). These bioactive plants-derived compounds could offer rich and powerful sources of therapeutic leads for the drug development (169, 171), and provide effective alternative strategies because of the better understanding of their cellular targets and mechanisms of action (170). Over the course of last decade, several

natural compounds have shown significant therapeutic efficacy in clinical trials conducted on cancer patients as indicated in Table 2.

Garcinia indica, commonly known as Mangosteen, presents many culinary, pharmaceutical and industrial uses (172). The spice kokum which is derived from this plant has been used traditionally in tropical areas as food ingredient, and also as Ayurvedic medicine, for the treatment of diseases as diverse as rheumatism, edema, ulcer and infectious diseases (173). Garcinol, a poly-isoprenylated benzophenone, is one of the major active compounds extracted from the dried rind of the fruit of *Garcinia indica* tree, has been reported to have anti-oxidative, anti-ulcer, anti- microbial, and anti-inflammatory activities (174-177). Along with the above effects, garcinol exhibits significant anti-neoplastic and chemopreventive roles in a variety of tumor cell lines and *in vivo* cancer models, identified by increasing number of studies in recent years.

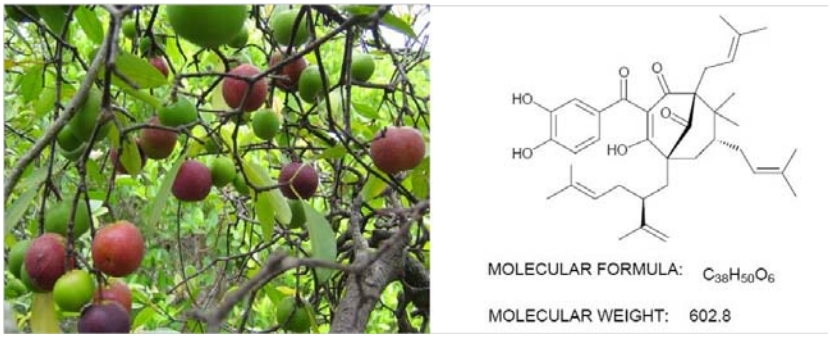


Figure 1.1. Fruit of *Garcinia indica* (left panel) and the chemical structure of garcinol (right panel)

1.3.1 Major molecular targets of garcinol

1.3.1.1 CBP/p300 Histone acetyltransferase

Histone acetyltransferase (HATs) refers to a group of enzymes which transfer an acetyl group from acetyl-CoA onto lysine residues on histones. Acetylation results in the neutralization of conserved lysine residues on the N-terminal tails of the core histones, that leads to the changes of histone: DNA and histone:histone interactions (178). Such conformational alteration renders DNA more accessible to transcriptional regulators (179). HATs work in concert with histone deacetylases (HDACs) that interplay with other regulators to maintain histone acetylation levels, and the acetylation defects owing to the altered HAT and HDAC activities can lead to several diseases, including cancer (180, 181). Besides the intrinsic acetyltransferase role, the major HATs cyclic AMP response element-binding protein (CBP)/p300 family proteins, function as factor acetyltransferases (FATs) to participate in transcriptional regulation by acetylating transcription factors such as p53, GATA-1 and c-Myb (182, 183). CBP/p300 has been found to be associated with tumor suppression which evidenced by the growth suppression functions of CBP/p300, loss or deregulation of CBP/p300 activity in various tumors and its role in maintaining the proper

functioning of other tumor-suppressor proteins (184, 185). However, the tumor-suppressor function of CBP/p300 has been questioned by the observation of proliferation defects in CBP/p300 null cells, activation of senescent or cell cycle arrest in the absence of CBP/p300 and its contribution to aberrant growth in certain leukemias through gain of function mutations (185-187).

Balasubramanyam *et al*. has demonstrated the garcinol can specifically inhibit the histone acetylation by p300 and p300/CBP Associated Factor (PCAF) *in vitro* as well as *in vivo* in Hela cells (188). The inhibition of acetylation *in vivo* by garcinol altered global gene expression with majority down-regulated, further microarray analysis revealed that garcinol repressed the expression of several proto-oncogenes, suggesting that garcinol may function as an anti-cancer compound. Garcinol is able to reprogram key histone and p53 post-translational modifications (PTMs) via modulating the expression of chromatin modifying enzymes in MCF7 cells (189). The garcinol-induced p300 degradation led to forkhead box P3 (FOXP3) hypo-acetylation, implying that garcinol could act as a potential inhibitor of FOXP3 and T_{reg} functions for cancer therapies (190).

1.3.1.2 NF-κB

Inflammation is a critical component of neoplastic progression that contributes to multiple aspects of tumor development including proliferation, survival and migration (191), and NF-κB is identified as a key mediator of inflammation-induced tumor growth and progression (108). The anti-inflammatory effects of garcinol has been evaluated in various inflammatory and tumor models and have been found to be mediated through the suppression of NF-κB activation. In an early study, garcinol has been found to suppress LPS and IFN-γ induced inflammatory mediators such as inducible nitric oxide synthase (iNOS) and COX-2 in macrophage RAW 264.7 cells (192), and such anti-inflammatory effect of garcinol was attributed to the inhibition of LPS-induced NF-κB activation (177). Several lysine residues on p65 could undergo acetylation modification by t he coactivator of p300 or PCAF thereby regulating NF-κB activation (193-195). As a naturally occurring cell-permeable HAT inhibitor, it is not surprising that garcinol has been found to significantly reduced the acetylation level of NF-κB, but has no obvi ous effects on the two NF-κB-regulated pro-inflammatory cytokines TNF-α and IL-6 (196). However, it has been reported recently that garcinol significantly decreased the pro-inflammatory mRNA

expression of COX-2, iNOS and IL-6, and IL-6 secretion by s uppressing phosphorylation of IκBα and p65 (197). It has been also found that garcinol inhibits cell proliferation and promotes apoptosis through the negative regulation of NF-κB signaling cascade in various types of cancers, such as breast cancer, pancreatic adenocarcinoma, and prostate cancer (198-200). Garcinol also causes reversal of epithelial-mesenchymal transition (EMT) to mesenchymal-epithelial transition (MET) and inhibits the invasion capacity of invasive breast cancer cells MDA-MB-231 and BT-549, and forced overexpression of NF-κB abrogates the garcinol-induced MET, induction of apoptosis, and inhibition of invasion (201). The detailed molecular mechanism(s) underlying garcinol-modulated NF-κB activation are not clear yet. However, it has been proposed that garcinol interrupts LPS binding to its toll-like receptors, thus affecting the downstream molecules in NF-κB activation cascade (202). Another study has suggested that garcinol blocks NF-κB translocation through suppressing the phosphorylation of IκBα mediated by p38 MAPK inhibition and anti-oxidant activity (177).

1.3.1.3 STATs

Garcinol has been found to modulate STATs signaling pathways in several studies. High interaction affinity of garcinol towards STAT1 protein has been demonstrated using surface plasmon resonance and molecular docking studies (203). Garcinol is able to inhibit IFN-γ-induced STAT1 activation, nuclear translocation and DNA binding activity in MDA-MB-231 cell (203). Similarly, garcinol suppresses the iNOS expression and nitric oxide (NO) by inhibition of Janus kinase (JAK)/STAT1 signaling in RAW 264.7 cell (202). Ahmad and his colleagues have reported the STAT3 inhibitory effects of garcinol in breast, prostate and pancreatic cancers (204). However, the in depth molecular mechanism(s) by which garcinol modulates STAT3 signaling pathway is not known yet.

1.3.1.4 Arachidonic acid metabolism

Aberrant arachidonic acid metabolism is involved in inflammation and carcinogenesis owing to the tumor promoting activities of the metabolic products prostaglandins (PGs) and leukotrienes (205, 206). Pharmacological intervention in the biosynthesis of these metabolites by inhibiting the relevant enzymes cytosolic

phospholipase A_2 ($cPLA_2$), COXs and lipoxygenases (LOXs) has been considered an effective approach for cancer chemoprevention and treatment (207-209).

Garcinol has been shown to significantly inhibit the release of arachidonic acid and its metabolites in macrophages by retarding the phosphorylation of $cPLA_2$ and suppressing COX-2 expression (202). However, Koeberlea and coworkers have demonstrated that garcinol selectively inhibits the activity of COX-1, with no significant reducing of COX-2 activity (210). They also found that garcinol potently interferes with 5-LOX and microsomal prostaglandin E synthase-1 (mPGES-1) to inhibit the enzymatic activities and biosynthesis of their enzymatic products leukotriene and PGE2 in cell-free system as well as in A549 human lung carcinoma cells (210). In a later study, using computer modeling analysis it was found that garcinol could fit well into the active site of 5-LOX, and potentially inhibit the enzyme activity through interactions between the phenolic hydroxyl groups and the non-heme catalytic iron (211). Chen and coworkers further tested the 5-LOX inhibitory activities of garcinol in DMBA-treated hamster cheek pouch model and showed that garcinol suppressed leukotriene B4 biosynthesis, inhibited inflammation, and reduced the number and size of carcinogen induced-tumors (211).

1.3.1.5 Other important molecular targets modulated by garcinol

MAPK signaling pathways are evolutionarily conserved signaling molecules that regulate the fundamental cellular processes such as cell growth, proliferation, differentiation, migration and apoptosis in response to stress stimuli and inflammation (212). Despite the negative regulation role of p38-MAPK in cell cycle progression (213), emerging evidences show that p38 a ctivation positively regulates proliferation, leads to increased survival and mediates metastasis in malignant cells; and several p38 inhibitors are effective in animal models and have advanced to clinical trials for the treatment of cancer (214). It is reported that garcinol significantly suppressed the activation of p38-MAPK, leading to the abrogation of inflammatory response of genes depending on NF-κB activation in macrophages (177). In lung cancer cell H1299, garcinol-induced inhibition of p38-MAPK activation upregulated $p21^{Waf1/Cip1}$ expression, resulted in G1 cell cycle arrest (215).

Altered activity of an important mediator of growth factor signaling, cell proliferation, survival and migration, focal-adhesion kinase (FAK), usually contributes to the development of various malignancies (216). Garcinol has been shown to suppress the activity of FAK signaling in human colon carcinoma cell

HT-29 and subsequent alteration of Bcl-2/Bax ratio and reduction of proteolytically active matrilysin (MMP-7) which accounts for its ability to induce apoptosis and inhibit cellular invasion (217).

MicroRNAs (miRNAs) are a class of small RNA (similar to 22 nucleotides) molecules which do not encode protein, but function directly as regulatory RNAs in various aspects of animal development through post-transcriptional modification (218). Number of these evolutionarily conserved miRNAs have been shown to modulate mammalian cell growth, differentiation and apoptosis (219), and implicated to act as oncogenes and/or tumor suppressors regulating these processes in various cancers (220). The garcinol mediated mesenchymal-epithelial transition observed in aggressive breast cancer has been associated with the upregulation of miR-200 and let-7 family miRNAs upon garcinol treatment (201). In a recent study, garcinol alone or in combination with gemcitabine has been found to alter microRNA profile in human pancreatic cancer cell line Panc-1 that might target key cancer pathway signaling molecules (221).

Wnt/β-catenin signaling is essential for stem cell function and embryogenesis by r egulating the change of embryonic epithelial cells to mesenchymal derivatives (222), and perturbations in Wnt signaling promotes

tumor EMT during cancer progression (222, 223). Recently, garcinol has been found to upregulate glycogen synthase kinase-3β (GSK-3β), along with the increasing levels of β-catenin phosphorylation, resulting in the inhibition of nuclear translocation of this key mediator of the Wnt signal in breast cancer MDA-MB-231 and BT-549 cells (201).

Besides the nervous system, nicotinic acetylcholine receptors (nAChRs) have been found to be universally expressed in mammalian cells, including multiple types of cancer (224, 225). Emerging evidences have shown that altered signaling of nAChRs promotes smoking-induced cancer formation by regulating the release of growth, angiogenic and neurogenic factors and stimulating signal transductions (226). Chen and colleagues have demonstrated that garcinol attenuated the nicotine-induced human breast cancer cell proliferation through downregulation of the nicotinic receptor, AP-1 activation and cyclin D3 expression (227).

1.3.2 Anti-cancer effects of garcinol *in vitro*

1.3.2.1 Garcinol induces cell cycle arrest

Cancer cells possess altered cell cycle kinetics to sustain the uncontrolled growth, thus the therapeutic agents that can modulate cell cycle progression are of great potential for cancer treatment. Gacinol has been found to induce G0/G1 phase cell cycle arrest in MDA-MB-231 cells through suppressing nicotinic receptor and down-regulation of D-type cyclins (227). While garcinol-induced p53-independent G1 cell cycle arrest in H1299 cells is associated with the upregulation of CDK Inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (215). Garcinol has been shown to cause reduction in the numbers of actively replicating cells (S phase) and concomitant increase in the G1 population in MCF7 cell, possibly through the inhibition of CBP/p300, or other HATs required for progression through S phase (189). Garcinol also facilitates senescence of A549 lung carcinoma cells through inhibiting double-strand break repair, including non-homologous end joining (NHEJ), without impairing activation of the cell cycle checkpoint (228).

1.3.2.2 Garcinol induces apoptosis

It is now well established that apoptotic pathways contribute to the cytotoxic action of most chemotherapeutic drugs through either extrinsic pathway initiated by death receptors, or intrinsic pathway which involves disruption of the mitochondrial membrane and the release of mitochondrial proteins (229, 230). Garcinol engages both pathways to induce the programmed cell death in a variety of neoplastic cells (231, 232). Garcinol has been shown to induce both of the TNF-related apoptosis-inducing ligand (TRAIL), death receptor 4 (DR4) and DR5 in a wide variety of cells including colon, breast, prostate, kidney, leukemic, and esophageal cancer cells, and subsequent activation of caspase-8 and downstream caspase cascades and apoptosis (231). The above mentioned upregulation of DR4 and DR5 by garcinol was mediated by a substantial increase in reactive oxygen species (ROS) generation. Garcinol could also cause the accumulation of ROS, increase growth arrest and DNA damage-inducible gene 153 (GADD153) expression and further resulted in downstream apoptotic events in hepatocellular carcinoma Hep3B cells (233). However, in another study, garcinol has been shown to reduce the LPS-induced increase of intracellular ROS involved in the activation of NF-κB (177). In addition, garcinol downregulated various cell

survival proteins including survivin, Bcl-2, X-linked inhibitor of apoptosis protein (XIAP), and c-FLIP and disrupted mitochondrial homeostasis through cleavage of bid, increasing pro-apoptotic bax expression and cytochrome c release that further contributed to enhanced apoptosis (231). These findings are in line with the observations from several reports indicating that garcinol exerts its apoptosis-inducing effects in a wide range of tumor cells through possible garcinol-induced modulation of important signaling cascades such as FAK, MAPK/ extracellular signal-regulated kinase (ERK), PI3K/Akt, NF-κB and JAK/STAT and their regulated gene products (198, 217, 221, 234).

1.3.2.3 Garcinol potentiates the effects of chemotherapeutic agents

Drug combination therapies are emerging as p romising strategies to improve therapeutic efficacy, tackle the problem of drug-related toxicity and major side effects, and overcome the chemoresistance (235, 236). Garcinol has been tested in combination with multiple agents in different tumor cells and displayed enhanced effect (221, 231, 237). Garcinol sensitized human pancreatic adenocarcinoma cells to the standard clinical chemotherapeutic agent gemcitabine that attenuated the drug-resistance phenotype (221). The same group has applied

garcinol together with another widely studied natural product curcumin, and observed synergistic effects in reduction of cell viability and increase of apoptosis in BxPC-3 and Panc-1 cells (237). Agonistic monoclonal antibodies to the TRAIL receptors and recombinant TRAIL are being developed in clinical trials as TRAIL selectively activate death receptors and induces apoptosis of a variety of tumor cells (238, 239). Prasad and his group have reported that garcinol could potentiate TRAIL-induced apoptosis in colon cancer HCT116 cells and sensitize TRAIL-resistant HT29 cancer cells to TRAIL (231).

1.3.2.4 Garcinol potentiates the effects of radiotherapy

The widely used ionizing radiation (IR) in the treatment of human cancers exerts the cytotoxic effects by producing DNA double-strand breaks, but usually the IR-induced double-strand breaks are preferentially repaired by non-homologous end joining that confers chemoresistance (240, 241). Garcinol specifically sensitizes cancer cells to radiotherapy with dose enhancement ratios by inhibiting NHEJ and also augments IR-induced senescence (228). In a study, it has been found that garcinol presents strong radiosensitizing effect comparable to the well-known radiosensitizers that target DNA repair proteins, such as poly

(ADP-ribose) polymerase (PARP) inhibitor olaparib, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) inhibitor NU7026 (242).

1.3.2.5 Garcinol inhibits tumor cell invasion and migration

The ability of garcinol to impede cell invasive capacity and migration has been reported in different cell lines and linked to several cancer signaling molecules. Garcinol inhibited cellular invasion in HT-29 cells through reduction of FAK tyrosine phosphorylation, and suppressing the downstream pathways for proteins associated with the focal adhesion complex (217). The reduced invasion and aggressiveness of cells after garcinol treatment have been observed in other tumor cell lines such as breast cancer (MDA-MB-231), prostate cancer (DU145) and pancreatic cancer (BxPC-3), and these effect have been found to be associated with garcinol-induced inhibition of STAT3 signaling and downregulation of its downstream targets urokinase-type plasminogen activator (uPA) and MMP-9 (204). Ahmad and his coworker suggested the garcinol-induced inhibition of invasion in aggressive breast cancer cells MDA-MB-231 was due to reversal of EMT phenotype which is mechanistically linked with the deregulation of miR-200s, let-7s, NF-κB, and Wnt signaling pathways (201). In

addition to invasion, garcinol also hindered migratory potential of BxPC-3 and Panc-1 cells, by suppressing NF-κB mediated MMP-9, IL-8 and PGE2 expression (199). Although there are no concrete evidence(s) to show that garcinol can also modulate neovascularization, several studies have reported that garcinol donwregulated the expression of key angiogenic factor VEGF, suggesting the anti-angiogenic potential of garcinol (199, 204, 221, 234).

1.3.3 Anti-cancer effects of garcinol *in vivo*

Pre-clinical animal models are indispensable and invaluable tools in predicting drug efficacy and toxicity in drug development. There are several reported *in vivo* studies elaborating the potential therapeutic effects of garcinol in diverse animal models.

1.3.3.1 Garcinol inhibits tumor growth in vivo

Immunohistochemical analysis of tumor tissues from garcinol treated breast cancer cell xenograft mice revealed that garcinol inhibited the tumor cell proliferation and microvessel density as evidenced by the downregulation of Ki-67 and CD31 expression (201). The same group found that garcinol can

significantly reduce the tumor growth in mice MDA-MB-231 cells xenografts (204).

1.3.3.2 Garcinol enhances the anti-tumor effects of targeted therapies in vivo

Garcinol has also been evaluated in drug combination study using syngeneic tumor mouse model. Garcinol was observed to enhance the *in vivo* anti-tumor activity of a targeted therapeutic anti-$p185^{her2/neu}$ antibody in BALB/c mice implanted with neu transformed mouse breast tumor cells H2N113, while either agent alone only modestly reduced the tumor growth (190).

1.3.3.3 Garcinol as a cancer chemopreventive agent in vivo

A number of dietary phytochemicals are being studied and evaluated in intervention trials for chemoprevention of cancers, as an inexpensive, readily applicable, acceptable and accessible approach to cancer control and management (243, 244). Garcinol has been investigated for its chemopreventive potential in several chemically-induced cancer models. Dietary administration of garcinol caused significant reduction in the frequency of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) in male F344 rats (192). Garcinol also exhibited

chemopreventive potential against HNSCC *in vivo*, specifically, dietary garcinol significantly decreased the incidence and multiplicity of 4-NQO-induced tongue neoplasms and/or pre-neoplasms in F344 rats(245), and topical application of garcinol on DMBA-treated hamster cheek pouch significantly reduced the size of visible tumors, and the number of cancer lesions (211).

1.3.4 Pharmacokinetic studies with garcinol

No prior reports have conducted detailed pharmacokinetic studies of garcinol in animals. Only one study has indicated that peak concentration of garcinol reached 12 and 2.7 μ M in plasma and urine respectively, after oral gavage of garcinol (10 mg dose per mouse) into CD-1 female mice (202).

1.3.5 Toxicological analysis of garcinol

Garcinol has been tested in several animal models through different administration modes including intraperitoneal injection, oral gavage, topical application and dietary administration (190, 204, 211, 245). So far, no obvi ous toxic effects caused by garcinol in these models have been reported as evidenced by the absence of body w eight loss and pathological alterations in important

organs such as liver, kidney, lung and heart (192, 211, 245). However, no systematic toxicological analysis of garcinol has been conducted yet.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents and chemicals

Cisplatin, dimethyl sulfoxide (DMSO), thiazoyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), dimethylformamide (DMF), propidium iodide (PI), sodium chloride (NaCl), HEPES, EDTA, triton X-100, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), sodium pervanadate (Na_3VO_4), tris, glycine, bovine serum albumin (BSA), β-actin antibody, acetone, 4',6-diamidino-2-phenylindole (DAPI), and mounting medium were bought from Sigma-Aldrich Co. (St. Louis, MO, USA). Garcinol with high purity (> 98%) was extracted from the rind of *Garcinia indica* fruit as described previously (188). Dulbecco's modified Eagle medium (DMEM), DMEM/F12, fetal bovine serum (FBS), L-glutamine, non-essential amino acid (NEAA), sodium pyruvate, vitamins, antibiotic-antimycotic mixture, trypsin, trypan blue vital stain, Lipofectamine® 2000, TRIzol ® reagent, Alexa Fluor® 594 goat anti-rabbit immunoglobulin G (IgG), and Click-iT® Plus EdU Flow Cytometry Assay Kit were purchased from Life Technologies (Carlsbad, CA, USA). Annexin V-FITC assay kit, antibodies against phospho-specific STAT3 (Ser 727), caspase-3, PARP,

cyclin D1, cyclin E, p27, Mcl-1, Bcl-xL, Bcl-2, survivin, $FLIP_{S/L}$, Bak, Bax, VEGF, MMP-2, ICAM-1, SHP2, PTP1B, PTEN, p65, IκBα, GAPDH, COX-2, Ki-67, goat anti-mouse HRP secondary antibody, and goat anti rabbit HRP conjugate were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies against phospho-specific STAT3 (Tyr705), phospho-specific JAK1 (Tyr 1022/1023), phospho-specific JAK2 (Tyr1007/1008), phospho-specific Src (Tyr416), phospho-specific p65 (Ser536), phospho-specific IκBα (Ser 32), phospho-specific TAK1 (Thr 187), phospho-specific IKKα/β (Ser 180 /Ser 181), phospho-specific mTOR (Ser2448), phospho-specific AKT (Ser 473), phospho-specific p70S6K (Thr 389), XIAP, caspase-8, caspase-9, MMP-9, STAT3, SHP1, Src, JAK1, JAK2, lamin B1, TAK1, IKKα, mTOR, AKT, p70S6K, and CD31 were obtained from Cell Signaling Technology (Danvers, MA, USA). Phospho-specific SHP1 (S591), phospho-specific SHP1 (Y536) and CXCR4 antibodies were purchased from Abcam (Cambridge, MA, USA). The cell Death Detection $ELISA^{PLUS}$ Kit was obtained from Roche Diagnostics (Indianapolis, IN, USA). Recombinant human IL-6, EGF, and CXCL12 (stromal cell-derived factor-1; SDF-1) were purchased from ProSpec (Israel). Bradford reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Blocking One reagent was

purchased from Nacalai Tesque (Kyoto, Japan). Western Bright Sirius HRP substrate was purchased from Advansta (Menlo Park, CA, USA). Bright-Glo™ Luciferase Assay System was purchased Promega (Madison, WI, USA). Trans AM™ nuclear extraction kit and STAT3/NF-κB Transcription Factor DNA-binding Kits were purchased from Active Motif (Carlsbad, CA, USA). Primers and probes for human *Bcl-2*, *Mcl-1*, *Bcl-xL*, *survivin*, *VEGF*, and *cyclin D1* were obtained as kits from Applied Biosystems® Life Technologies. The immunohistochemistry (IHC) DAKO LSAB kit was purchased from Dako Corporation (Carpinteria, CA, USA).

2.1.2 Cell lines

Human HNSCC cell line UMSCC1 was kindly provided by Prof. Thomas E. Carey (University of Michigan, Ann Arbor, MI, USA) and have been characterized previously. MDA686Tu, MDA686LN, and Tu138 were kindly provided to us by Prof. Jeffrey N. Myers (The University of Texas MD Anderson Cancer Center, Houston, Texas, USA) and have been characterized previously. CAL27 was purchased from American Type Culture Collection. UMSCC1 and CAL27 were cultured in DMEM supplemented with 100 U/mL penicillin, 100

μg/mL streptomycin and 10% FBS. MDA686Tu and MDA686LN were cultured in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% FBS, 2mM glutamine 1× NEAA, 1× sodium pyruvate, and 1× vitamin. Tu138 was cultured in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. All the cell lines used were grown and passaged in a humidified incubator at 37°C with 5% CO_2.

2.2 Methods

2.2.1 MTT cell viability assay

MTT assay was used to examine the effect of garcinol on HNSCC cell viability. Briefly HNSCC cells (1×10^4/mL) in a volume of 200 μL were incubated in 96-well plates with different concentrations for the indicated time points. Then, 20 μL of MTT solution (5 mg/mL) was added, after which the plates were incubated for 2 h at 37˚C to allow converting of MTT to its insoluble purple formazan by NAD(P)H-dependent cellular oxidoreductase enzymes in metabolically intact cells. After removal of the medium, the purple formazan crystals formed were dissolved in 100 μL lysis buffer (50% DMF, 20% SDS) with 1h incubation at 37˚C. The optical density (OD) of dissolved purple crystal was measured by Safire2™ microplate reader (Tecan Group, Männedorf, Switzerland) at 570 nm.

2.2.2 EdU cell proliferation assay

Click-iT® Plus EdU Flow Cytometry Assay Kit (Life Technologies, Carlsbad, CA, USA) was used to measure HNSCC cell's ability to proliferate with or without garcinol treatment. Briefly, CAL27 cells (5×10^5/mL) were treated

with 15 μM and 25 μM garcinol for 12, 24, and 48 h. The cells were pulse labeled with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU; thymidine analog) for 2 h, thereafter cells were trypsinized, washed with PBS, and fixed with Click-iT® fixative. After washing with PBS, the cells were permeabilized with Click-iT® saponin-based permeabilization and wash reagent. Then, the Click-iT reaction was allowed after incubation with Click-iT® Plus reaction cocktail to label the EdU- incorporated cells with Alexa Fluor® 488 dye. Standard flow cytometry method was used for determining the percentage of S-phase cells in the population using CyAn™ ADP Analyzer (Dako Corporation, Carpinteria, CA, USA).

2.2.3 Cell cycle analysis by flow cytometry

HNSCC cells (5×10^5/mL) were seeded and then synchronized by starvation in serum-free medium for 24 h. Upon completion of 25 μM garcinol treatment for 12, 24, and 48 h, the cells were collected following trypsinization, and then fixed with ice-cold 70% ethanol for 30 min. The fixed cells were washed with PBS, treated with RNase A (1 μg/mL), and stained with propidium iodide

(10 μg/mL) protected from light for 30 min. DNA contents of the samples were analyzed using CyAn™ ADP Analyzer.

2.2.4 Enzyme-linked immunosorbent assay (ELISA) for DNA fragmentation detection

Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Indianapolis, IN, USA) was used for DNA fragmentation detection as described previously. Briefly, HNSCC cells (5×10^5/mL) were exposed to 25 μM garcinol for 12, 24, and 48 h. After the incubation, the cytoplasmic fractions were extracted and transferred into the streptavidin-coated microplate incubated with the biotinylated histone antibody and anti-DNA-peroxidase conjugate for 2 h at room temperature. The photometric signals were determined by measuring absorbance at 405 nm after adding ABTS substrate. The enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated as absorbance of sample cells/absorbance of control cells.

2.2.5 Apoptosis detection by Annexin V staining

The apoptosis inducing effect was further evaluated by Annexin V-FITC assay kit. Briefly, HNSCC cells (5×10^5/mL) were exposed to 25 μM garcinol for 12, 24, and 48 h, and then harvested and washed with PBS. The prepared cells were resuspended in binding buffer followed by incubation with annexin V-FITC and PI for 15 min in darkness. The apoptotic index was immediately determined by flow cytometry using CyAn™ ADP Analyzer.

2.2.6 Western blot analysis

To prepare the whole cell protein extracts, cells were collected by scraping, and then lysed in lysis buffer containing NaCl (250 mM), EDTA (2 mM, pH 8.0), EGTA (0.5 mM), HEPES (20 mM), 0.1% Triton X-100, aprotinin (1.5 μg/mL), leupeptin (1.5 μg/mL), PMSF (1 mM), and sodium orthovanadate (1.5 mM). After incubation on ice for 15 min, lysed cells were then centrifuged at 13,300 rpm for 10 min to pellet debris. Cellular proteins were separated on 8%, 10%, 12%, or 15% SDS-PAGE gel, and then transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were placed in blocking buffer (Blocking One reagent) for 1 h, followed by incubation with primary

antibodies overnight at 4˚C. Appropriate secondary antibodies conjugated to streptavidin-HRP were used for signal detection, upon membrane incubation with Western Bright Sirius HRP substrate and detection by Molecular Imager® ChemiDoc™ XRS System (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analysis of Western blots was performed using Image J software and band intensity was normalized to untreated control as fold change.

2.2.7 Immunocytochemistry for STAT3 localization

HNSCC cells grown in 8-well Nunc Lab-Tek Chamber Slide (Thermo Fisher Scientific, Waltham, MA, USA) were exposed to garcinol treatment as indicated. Thereafter, the slides were fixed with ice-cold acetone, permeabilized in 0.2% TritonX-100, followed by blocking in 5% FBS. Primary antibody to STAT3 (1:100 dilution) was added onto the cells and left overnight at 4 °C. Cells were subsequently incubated with Alexa Fluor® 594 (1:100 dilution) secondary antibody for 1 h at room temperature. Staining with 0.5 μg/mL DAPI was done for visualization of the cell nuclei. After mounting, the immunofluorescence images were visualized using an Olympus DP 70 fluorescent microscope.

2.2.8 Preparation of cytoplasmic and nuclear extracts

Nuclear proteins were extracted from cell lines using Trans AM™ nuclear extraction kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were washed with phosphatase inhibitors-containing PBS and collected by s craping. After centrifugation at 500 rpm for 5 min, the supernatant was discarded and the cell pellet was resuspended with 1× Hypotonic Buffer which was then incubated on ice for 15 min. Detergent was added to the suspension and vortexed at highest setting for 10 s econds. Supernatant (cytoplasmic fraction) was collected after centrifugation and stored at -80˚C. The nuclear pellet was resuspended in complete lysis buffer by pipeting up and down. After vortexing for 10 seconds, the suspension was incubated for 30 min on ice on a rocking platform set at 150 rpm. Suspension was then vortexed again for 30 seconds and centrifuged at 14,000g for 10 min. Supernatant (nuclear fraction) was collected and stored at -80˚C until ready to use.

2.2.9 DNA-binding assay

The DNA-binding capacity of STAT3 or NF-κB was determined by using Trans AM™ STAT3 or NF-κB transcription factor assay kit (Active Motif,

Carlsbad, CA, USA) as described previously (234). Briefly, 20 µg of nuclear proteins were subjected to binding of STAT3 or NF-κB to an immobilized consensus sequence in a 9 6-well plate respectively, and primary and HRP-conjugated secondary antibodies were added. After incubating with substrate, the colorimetric reading at 450 nm was determined in a microplate reader. The experiments were carried out according to the manufacturer's instruction.

2.2.10 RNA extraction and real time PCR

RNA sample was isolated by TRIzol method. Briefly, after treatment with garcinol, HNSCC cells were subjected to homogenization in cold TRIzol reagent. Phase separation of the cell suspension was performed after the addition of chloroform, followed by RNA precipitation with isopropyl alcohol. The purified RNA in the pellet was washed with75 % ethanol, air dried, and dissolved in nuclease free water. Total RNA was reverse-transcribed into single-strand complementary DNA (cDNA), thereafter the real time PCR analyses were performed in duplicate with Applied Biosystems 7500 Fast Real-Time PCR System as described previously (234). Data was evaluated using Sequence

Detection Software (Applied Biosystems® Life Technologies), and the $2^{-\Delta\Delta Ct}$ formula.

2.2.11 STAT3 luciferase reporter assay

MDA686Tu (1×10^4 cells/well) in 96-well were co-transfected of plasmid with STAT3-responsive elements linked to a luciferase reporter gene and wild-type or dominant-negative STAT3 constrcut using lipofectamine® 2000. After transfection for 48 h, various concentrations of garcinol were added into the wells for 6 h, f ollowed by 6 h incubation with IL-6 or EGF. Luciferase activity was determined using Bright-Glo™ Luciferase Assay System from Promega (Madison, WI, USA).

2.2.12 Wound-healing migration assay

The migration of cancer cells was investigated by wound-healing assay using IBIDI culture insert (ibidi GmbH, Munich, Germany) consisting of two reservoirs separated by a 500 μm thick wall in a 35 mm petri dish. CAL27 cells were starved to inactivate cell proliferation and then an equal number of cells (70 μL; 1×10^6 cells/mL) were seeded into the two reservoirs of the same insert. After

12 h, the insert was gently removed creating a gap of ~500 μm. The cells were then treated with garcinol and or CXCL12 as indicated, after which the wound closure areas were visualized using bright field microscopy. Wound healing rate was calculated as the difference in wound area before and after treatment.

2.2.13 Invasion assay

BD BioCoat Matrigel Invasion Chamber (BD Biosciences, San Jose, CA, USA) assays were done in 24-well plates using polycarbonate inserts with Matrigel coating (8-μm pores) to examine *in vitro* cell invasion. A total 500 μL CAL27 cells (5×10^5 cells/mL) were seeded in the upper compartment of the transwell chamber, and treated with 15 μM garcinol for 8 h after which 100 ng/mL CXCL12 was added into the lower chamber of the 24-well plate for additional 24 h incubation. Non-invading cells on the upper side of the insert membrane were removed using a cotton swab, while invading cells attached to the bottom were fixed with 4% paraformaldehyde and stained with crystal violet. Multiple images were taken from randomly selected areas under microscopy and the invading cell numbers were counted.

2.2.14 Xenograft tumor model

Males, aged five weeks, nu/nu athymic mice (Biological Resource Centre, Biopolis, Singapore) were used as xenograft recipients for human HNSCC tumors. 2×10^6 CAL27 cells were resuspended in the saline in total volume of 100 μL and were then injected subcutaneously into the flank of the animals. When tumors reached 0.25 cm in diameter, animals were randomly divided into three experimental groups (n = 5, per group), specifically the vehicle-treated control and garcinol at a dose of 1 and 2 mg/kg (body weight). Garcinol dissolved in saline with 0.1% DMSO (100 μL, total volume) was injected in the mice intraperitoneally five times a week for 4 weeks, while control group received injections of 100 μL of vehicle saline buffer. Tumor volume and body weight of each mouse were evaluated three twice a week. The diameters of the tumor were measured with vernier caliper, after which volume of the tumor was calculated as: (longest diameter) × (shortest diameter)^2) × 0.5. Mice were sacrificed 4 weeks after initiating treatment, and tumors were excised and either stored at -80 °C for western blot or processed and embedded in paraffin for immunohistochemistry analysis. All the mouse experiments reported in this study were reviewed and approved by NUS Institutional Animal Care and Use Committee.

2.2.15 Immunohistochemical analysis of tumor tissues

Immunohistochemical analysis was carried out on formalin-fixed, paraffin-embedded sections of each tumor specimen using the streptavidin-biotin-peroxidase technique. Briefly, after deparaffinization in xylene, slides were rehydrated in graded ethanol and then heated for antigens retrieval, thereafter the slides were incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. After blocking in the blocking reagent, the sections were then incubated with appropriate primary antibodies (1:100 dilution) overnight, followed by incubation with biotinylated linker and then streptavidin-peroxidase complex at room temperature. Then the sections were incubated with 3, 30'-diaminobenzidine tetrahydrochloride (DAB) working solution for color reactions, and finally, counterstained with hematoxylin and mounted. Slides were visualized using Olympus BX51 microscope at ×20 magnification, and quantitative analysis of images was performed using Image-Pro plus software (Media Cybernetics, Rockville, USA).

2.2.16 Xenograft tumor model for drug combination study

Females, aged six weeks, nu/nu athymic mice (Animal Resource Centre, Australia) were used as xenograft recipients for human HNSCC tumors. 3×10^6 CAL27 cells were resuspended in the saline in total volume of 100 μL and were then injected subcutaneously into the flank of the animals. When tumors reached 0.25 cm in diameter, animals were randomly divided into five experimental groups (n = 5, per group), namely: (I) vehicle-treated control; (II) garcinol (0.5 mg/kg body weight); (III) cisplatin (2 mg/kg body weight); and (IV) combination of garcinol and cisplatin. Garcinol dissolved in saline with 0.1% DMSO (100 μL, total volume) was injected in the mice intraperitoneally five times a week, cisplatin in saline was injected in the mice intraperitoneally two times a week, while control group received injections of 100 μL of vehicle saline buffer. Tumor diameters and body weight of each mouse were measured three twice a week. Tumor volume was calculated using the formula: (longest diameter) × (shortest diameter) ^{2}) × 0.5. Mice were sacrificed 4 weeks after initiating treatment, and tumors were excised and either stored at -80 °C for western blot or processed and embedded in paraffin for immunohistochemistry analysis. All the

mouse experiments reported in this study were reviewed and approved by NUS Institutional Animal Care and Use Committee.

2.2.17 Pharmacokinetic study of garcinol in mice

Pharmacokinetics of garcinol was conducted in nude mice by i.p. injection at dose of 0.5 mg/kg or 2 mg/kg. The mice were allowed food and water ad libitum before pharmacokinetics experiment. About 150 μL of blood was taken from facial vein of mice at 10 min, 30 min, 1, 2, 4, 6, and 8 h postdose. After centrifugation of blood samples at 14,000 rpm at 4°C for 10 min, mouse sera (the supernatant) were transferred into eppendorf tube. 10 μL of each serum was processed by adding 3-folds excess of acetonitrile-containing isogarcinol, the internal standard followed by vortexing for 1 min. The tube was then centrifuged at 14,000 rpm at 4°C for 10 min. Then 30 μL of the supernatants were transferred into glass inserts (250 μL) with 70 μL of 10 mM ammonia acetate solution. 50 μL of processed serum samples were injected into LC-MS/MS which was carried out under negative electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. Mass spectra of garcinol and its internal standard, isogarcinol showed a same precursor ion $[M-H]^-$ at m/z of 601. The daughter ion monitored

for garcinol and isogarcinol was 409 and 335, respectively. SCIEX Analyst software (version 1.4.2) was used for data acquisition and analysis.

2.2.18 Statistical analysis

The results were expressed as the means±standard deviations. Quantitative variables were compared with Student's t-test, or analyses of variance (ANOVA). All p values were 2-tailed, and p value <0.05 was considered statistically significant.

3 RESULTS

3.1 Anti-cancer effects of garcinol on HNSCC cells *in vitro*

3.1.1 Garcinol inhibits the viability of HNSCC cells.

The cytotoxic ability of garcinol was assessed on a panel of diverse HNSCC cell lines by the MTT assay. The cells were exposed to various concentrations of garcinol for different time interals as indicated and then subjected to MTT assay. It is clear from our data that garcinol significantly inhibited the viability of CAL27, UMSCC1, MDA686LN, MDA686Tu, and Tu138 cell lines in a time- and dose-dependent manner.

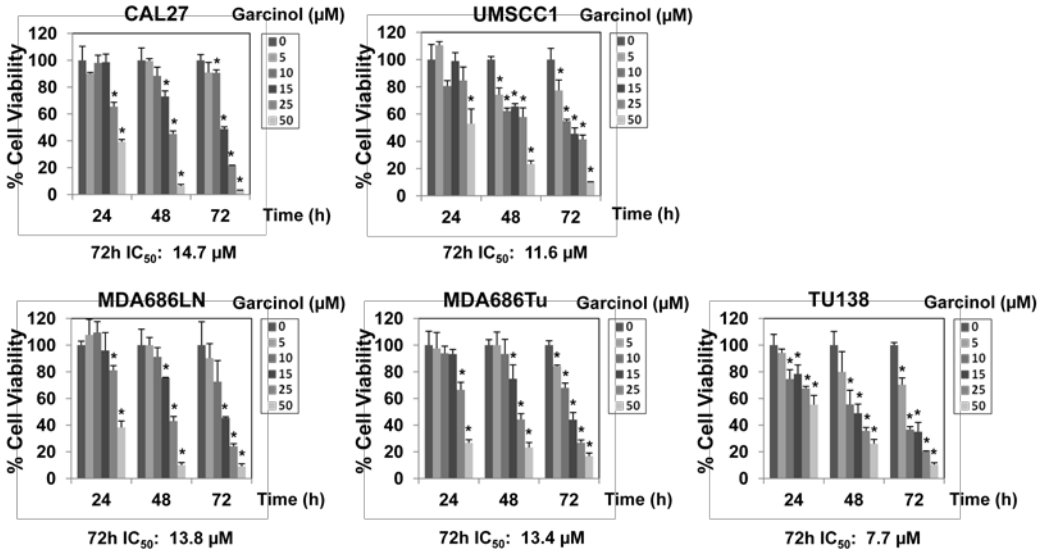


Figure 3.1.1. Garcinol suppresses the viability of various HNSCC cells.

CAL27, UMSCC1, MDA686LN, MDA686Tu, and Tu138 cells (1×10^4/mL) were exposed to 0, 5, 10, 15, 25, and 50 μM of garcinol for 24, 48, and 72 h. Cell viability was then analyzed by MTT assay. (*p <0.05)

3.1.2 Garcinol suppresses the cell proliferation of HNSCC cells.

The anti-proliferative effect of garcinol was further evaluated by nuclear EdU incorporation method to measure the DNA synthesis. CAL27 cells (5×10^5/mL) were exposed to 0, 15 and 25 μM garcinol for 12, 24, and 48 h, after which the cells were pulse labeled with EdU, a nucleoside analogue to thymidine which is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction, in which EdU is coupled to Alexa Fluor® 488 dye. The EdU-incorporated cells were then detected by flow cytometric analysis. We found that the percentage of S-phase cells decreased in a time- and dose-dependent manner, suggesting a reduction of active DNA synthesis and cell proliferation after garcinol treatment.

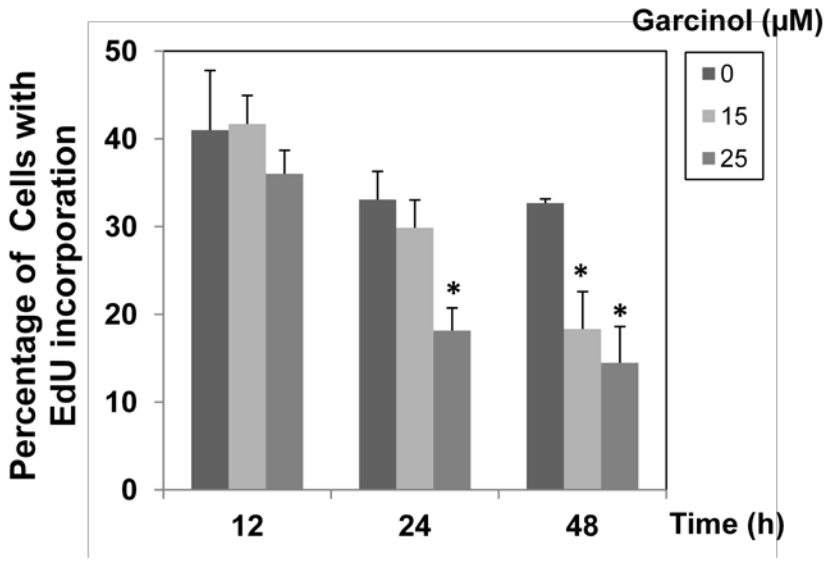


Figure 3.1.2. Garcinol inhibits cell proliferation of HNSCC cells.

CAL27 (5×10^5/mL) were treated with 0, 15, and 25 μM of garcinol for 12, 24, and 48 h, and then analyzed by Click-iT® Plus EdU Flow Cytometry Assay Kit. (*$p < 0.05$)

3.1.3 Garcinol induces apoptosis in HNSCC cells.

To investigate whether the inhibition of cell viability by garcinol is also due to its apoptotic effect, a variety of experiments were conducted in HNSCC cells.

3.1.3.1 Garcinol increases the population of annexin V-positive cells.

In the early stage of apoptosis, the membrane phospholipids as well as phosphatidylserines translocate rapidly from cytoplasmic interface and accumulate at extracellular surface (246). This process results in the loss of membrane asymmetry which can be detected using Annexin V. As a phospholipid binding protein, Annexin V preferentially binds to negatively charged phospholipids including phosphatidylserines (247). The Annexin V kit was utilized to monitor the progressing of early apoptosis in garcinol treated HNSCC cells according to their Annexin V and PI staining pattern. CAL27 and UMSCC1 cells (5×10^5/mL) were exposed to 25 μM garcinol for different time points as indicated, and then were harvested, stained with PI and FITC-conjugated annexin V for flow cytometric analysis. Our data indicated that Annexin V-positive and PI-positive cells increased progressively in a time-dependent manner upon garcinol treatment.

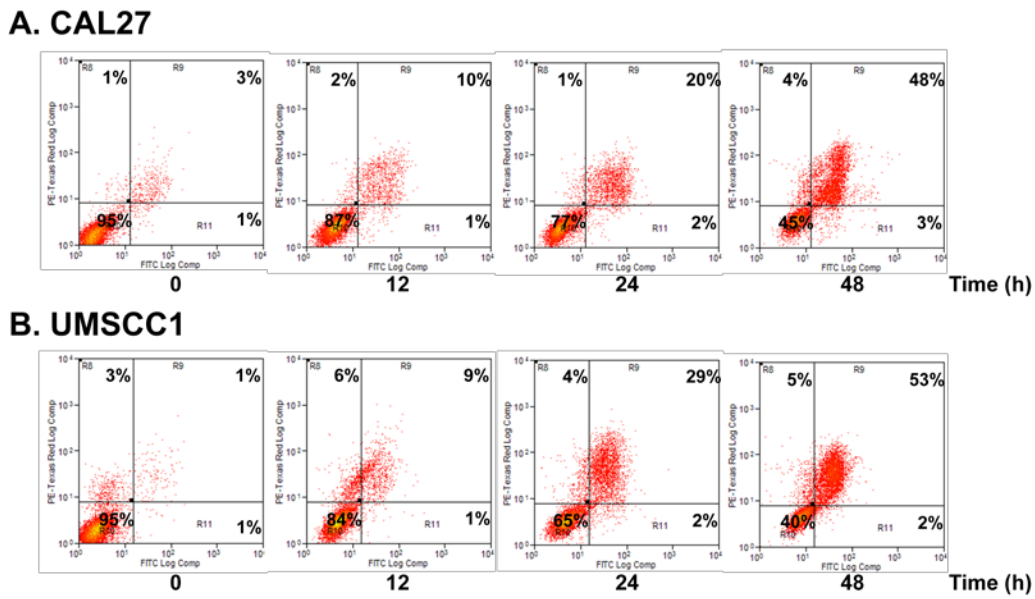


Figure 3.1.3.1. Garcinol increases the population of annexin V-positive cells in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 12, 24, and 48 h, the cells were then harvested and stained with PI and annexin V, and analyzed by flow cytometry. Data representative of at least two independent experiments is shown.

B. UMSCC1 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 12, 24, and 48 h, the cells were then harvested and stained with PI and annexin V, and analyzed by flow cytometry. Data representative of at least two independent experiments is shown.

3.1.3.2 Garcinol increases the accumulation of HNSCC cells in Sub-G1 phase.

DNA fragmentation is a key feature associated with apoptosis, and the loss of DNA can be detected from ethanol permeabilized cells due to DNA fragmentation and presents as an accumulation of Sub-G1 population in flow cytometric analysis after PI staining (248). CAL27 and UMSCC1 cells (5×10^5/mL) were incubated with 25 μM garcinol for different time points as indicated, and then were collected and stained with PI, and analyzed for DNA contents by fluorescence-activated cell sorting (FACS). We observed that garcinol could caused an increased accumulation of cells in Sub-G1 phase in a time-dependent manner in both the cell lines tested.

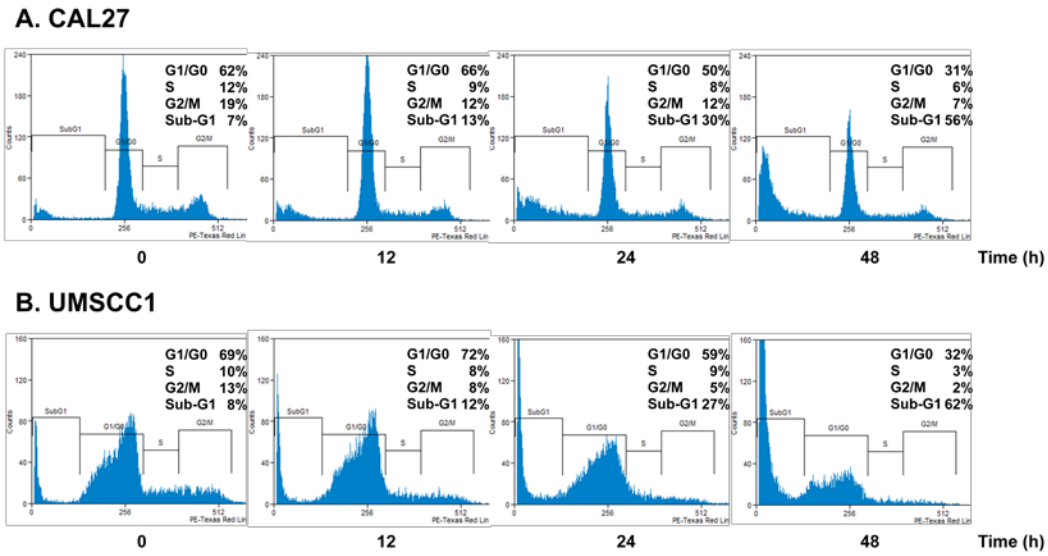


Figure 3.1.3.2. Garcinol increases the accumulation of HNSCC cells in Sub-G1 phase.

A. CAL27 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 12, 24, and 48 h, then the cells were fixed by 70% ethanol, stained with PI, and subjected to FACS. Data representative of at least two independent experiments is shown.

B. UMSCC1 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 12, 24, and 48 h, then the cells were fixed by 70% ethanol, stained with PI, and subjected to FACS. Data representative of at least two independent experiments is shown.

3.1.3.3 Garcinol induces DNA fragmentation of HNSCC cells.

The activation of endogenous endonucleases during apoptosis cleave double stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes (249). The use of Cell Death Detection ELISA^{PLUS} kit allows the specific determination of the enrichment of these degraded DNA fragments in the cytoplasm of cells. The cytoplasmic fraction of the cells was extracted after exposure of CAL27 cell to 25 μM garcinol for the indicated time intervals. The cytoplasmic lysates were subjected to the quantitative enzyme-immunoassay. The data revealed that garcinol significantly increased the DNA fragmentation level in a time-dependent manner in CAL27 cells which is an indicative of apoptosis.

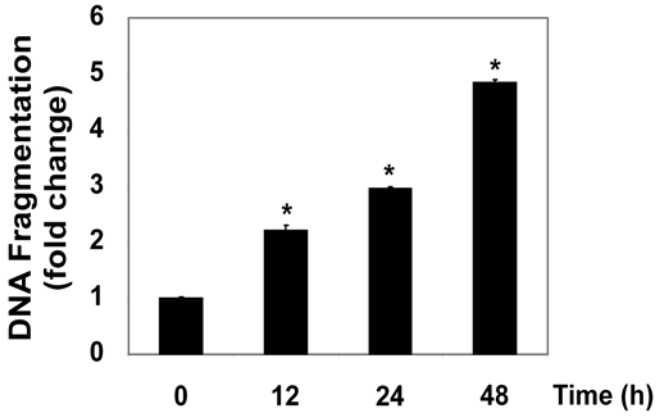


Figure 3.1.3.3. Garcinol induces DNA fragmentation in HNSCC cells.

CAL27 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 12, 24, and 48 h, after which the cytoplasmic fractions were collected and analyzed by Cell Death Detection ELISA^{PLUS} kit to determine the degree of DNA. (*$p < 0.05$)

3.1.3.4 Garcinol activates caspase-3 in HNSCC cells.

Cysteine-aspartic acid protease 3 (caspase-3) is one of the key executioners of apoptosis, upon activation, it proteolytically cleaves many key proteins, such as the nuclear enzyme PARP, which are cleaved in many different systems during apoptosis (250). HNSCC cells were exposed to 25 μM garcinol for various time intervals, after which the whole cell lysates were examined by western for caspase-3 expression. It is clear from our data that garcinol induced caspase-3 activation as evidenced by a time-dependent cleavage of caspase-3. It is also found that the activation of this effector caspase cleaved the full length PARP protein into smaller active fragment. These findings indicate that garcinol induces caspase-3-dependent apoptosis in HNSCC cells.

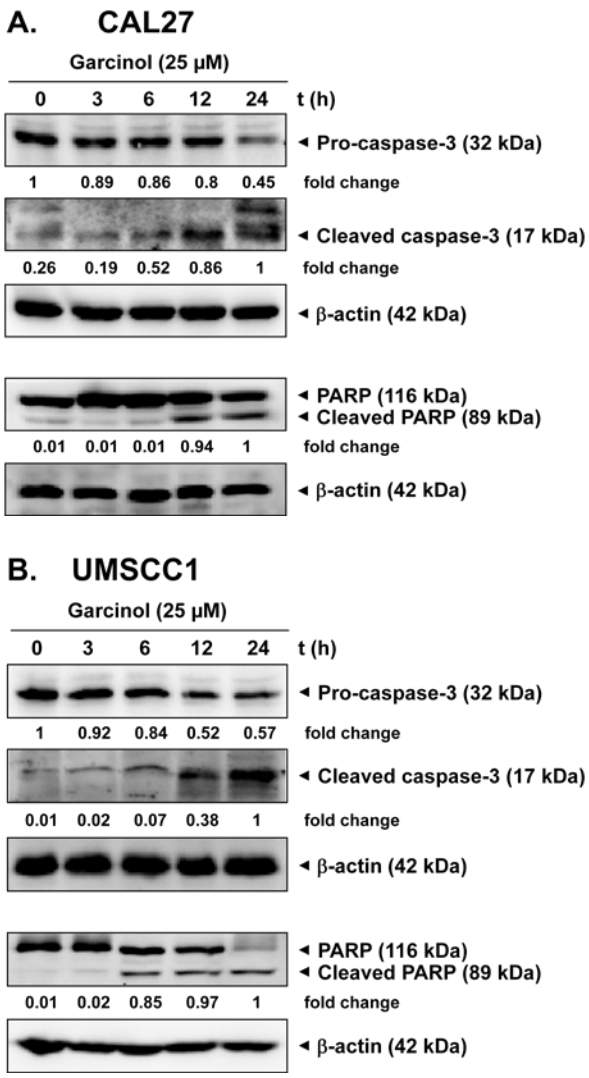


Figure 3.1.3.4. Garcinol activates caspase-3 in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24 h; whole cell proteins were extracted and analyzed by western blot using antibodies specific to caspase-3 and PARP. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Data representative of at least two independent experiments is shown.

B. UMSCC1 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24 h; whole cell proteins were extracted and analyzed by western blot using antibodies specific to caspase-3 and PARP. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Data representative of at least two independent experiments is shown.

3.1.3.5 Garcinol activates both intrinsic and extrinsic caspase activation cascades to induce apoptosis in HNSCC cells.

Current anti-cancer chemotherapies initiate a programmed cell death cascade engaging the activation of both intrinsic and extrinsic apoptosis signal transduction pathways in cancer cells (251). Western blot was used to explore the involvement of extrinsic and/ or intrinsic pathways in the garcinol-induced apoptosis. Whole cell lysates were extracted from HNSCC cells which were treated with 25 μM garcinol for 0, 3, 6, 12, and 24 h, and were then subjected to western blot to analyze the expression of caspase-8 and caspase-9. A time-dependent reduction of full length caspase-8 and caspase-9 were observed, with a corresponding increase in the expression level of cleaved caspase-8 and caspase-9, suggesting that garcinol-induced apoptosis in HNSCC cells is indeed mediated through both the extrinsic and intrinsic caspase activation cascades.

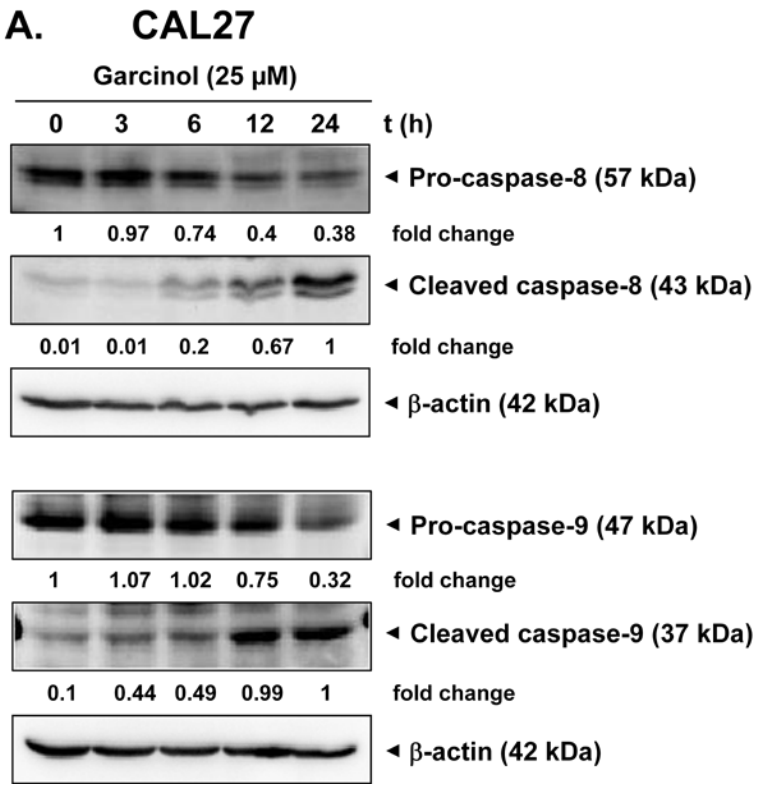


Figure 3.1.3.5. Garcinol causes the activation of both intrinsic and extrinsic caspase cascades in HNSCC cells.

After treated with 25 μM garcinol for 0, 3, 6, 12, a nd 24 h, t he protein levels of caspase-8 and caspase-9 in whole cell lysates were examined by western blot. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

3.1.3.6 Garcinol modulates the expression of various proteins involved in the cell cycle progression in HNSCC cells.

We next proceeded to investigate whether garcinol modulates the constitutive expression of various proteins involved in cell cycle progression. HNSCC cells were incubated with 25 μM garcinol for 0, 3, 6, 12, and 24 h, after which whole cell lysates were extracted. The expression of cyclin D1, cyclin E, and p27 were detected by w estern blot analysis. We found that garcinol downregulated the proteins involved in cell cycle progression namely cyclin D1 and cyclin E, while upregulated the expression of p27, a negative regulator of cell cycle progression, in a time-dependent manner.

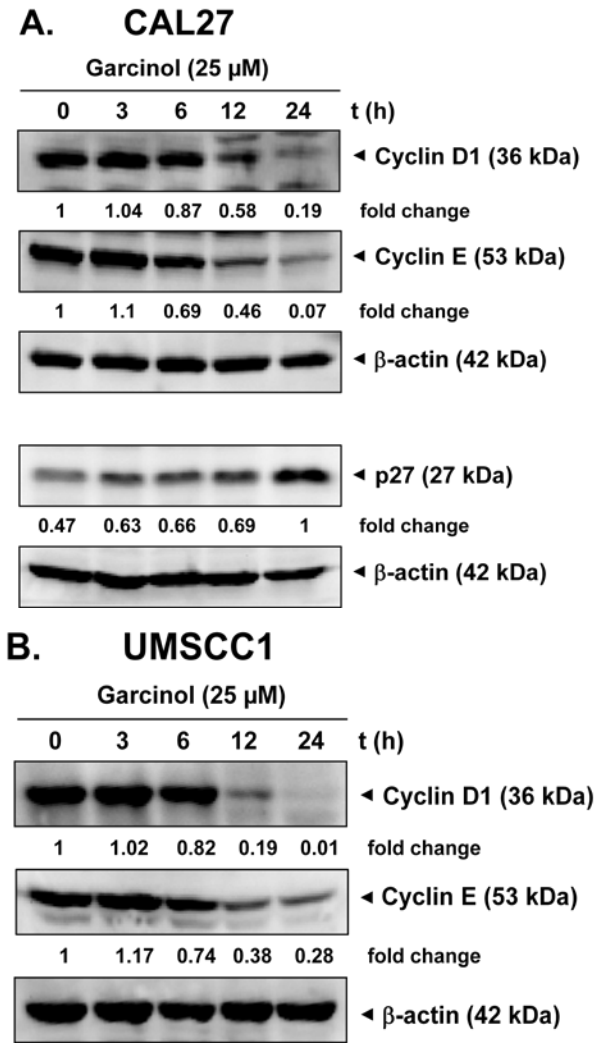


Figure 3.1.3.6. Garcinol modulates the expression of various proteins involved in the cell cycle progression in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24 h. Levels of cyclin D1, cyclin E, and p27 in whole cell lysates were analyzed by western blot. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

B. UMSCC1 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24 h. Levels of cyclin D1 and cyclin E in whole cell lysate were analyzed by western blot. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

3.1.3.7 Garcinol modulates the expression of various proteins involved in the survival/apoptosis of HNSCC cells.

The susceptibility of tumor cells to apoptosis induced by anti-tumor drugs depends on the balance between pro-apoptotic and survival (anti-apoptotic) signals (252). Next we investigated whether garcinol modulates the expression of pro-apoptotic and anti-apoptotic proteins that determine the ultimate fate of the tumor cells. HNSCC cells were exposed to 25 μM garcinol for different time intervals as indicated, whole cell lysates were then analyzed by western blot against proteins of interests. The results showed that garcinol downregulated the expression of several anti-apoptotic members of Bcl-2 family proteins namely Bcl-2, Bcl-xL and Mcl-1, as well as other inhibitors of apoptosis such as survivin, XIAP and c-FLIP. However, the expression of pro-apoptotic proteins namely (Bak, and Bax) largely remained unchanged after garcinol treatment.

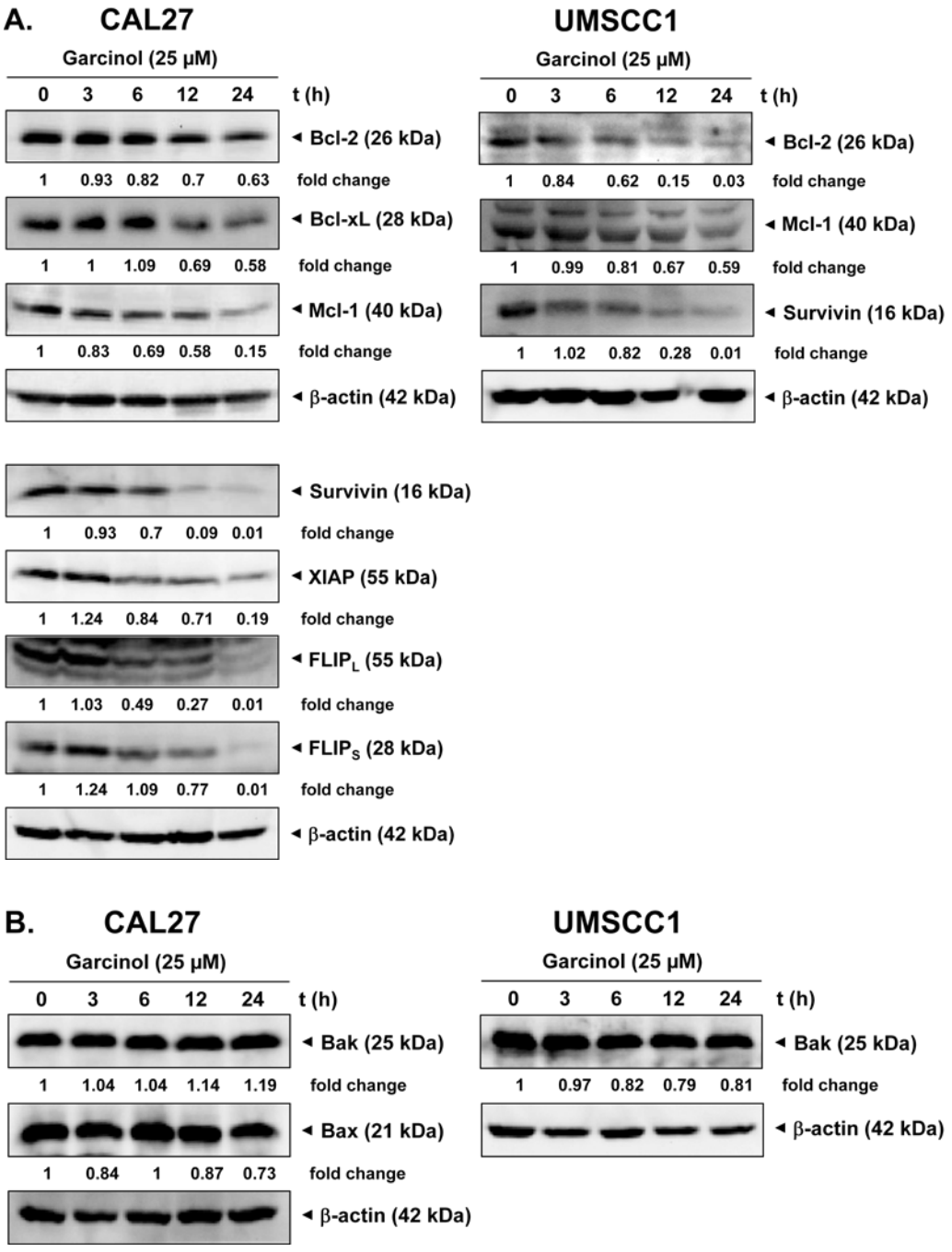


Figure 3.1.3.7. Garcinol modulates the expression of various proteins involved in the survival/apoptosis of HNSCC cells.

A. CAL27 (*right panel*) and UMSCC1 (*left panel*) cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24 h; whole cell lysates were extracted and subjected to western blot against pro-apoptotic proteins. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane

after stripping. Representative blots from at least two independent experiments have been shown.

B. CAL27 (*right panel*) and UMSCC1 (*left panel*) cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24 h; whole cell lysates were extracted and subjected to western blot against anti-apoptotic proteins. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

3.1.3.8 Garcinol donwregulates the transcription expression of various proteins involved in the cell proliferation and survival of HNSCC cells.

To determine whether garcinol also modulates the transcription of various cell cycle and survival genes, the mRNA expression levels of cyclin D1, Bcl-xL, Mcl-1, Bcl-2, and survivin were measured upon garcinol treatment. CAL27 and UMSCC1 cells were exposed to 25 μM garcinol for various time points as indicated, and RNA was extracted and subjected to real time PCR. We first noticed the constitutive mRNA expression of various oncogenic genes in HNSCC cells, and garcinol significantly inhibited the expression of these genes at transcriptional level in a time-dependent manner.

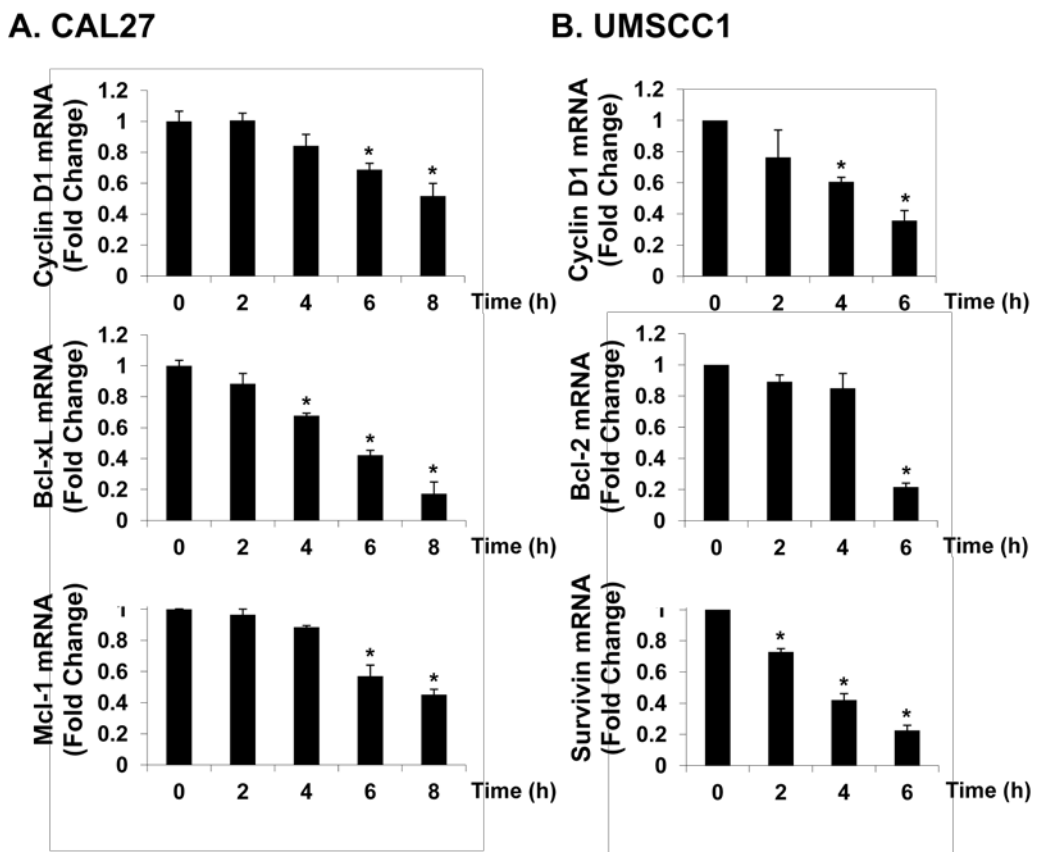


Figure 3.1.3.8. Garcinol donwregulates the transcription expression of various proteins involved in the cell proliferation and survival of HNSCC cells.

A. CAL27 cells (1×10^6/mL) were exposed to 25 μM garcinol for 0, 2, 4, 6, and 8 h. The cells were then harvested for RNA extraction, after which the relative expression of mRNA was evaluated by real time PCR. (*p <0.05)

B. UMSCC1 cells (1×10^6/mL) were exposed to 25 μM garcinol for 0, 2, 4, and 6 h. The cells were then harvested for RNA extraction, after which the relative expression of mRNA was evaluated by real time PCR. (*p <0.05)

3.1.4 Garcinol downregulates the expression of gene product involved in angiogenesis in HNSCC cells.

VEGF is the main angiogenic factor that is commonly overexpressed in variety of cancers including HNSCC (253, 254). To examine the effects of garcinol on VEGF, HNSCC cells were treated with 25 μM garcinol for various time points. Thereafter, protein levels of VEGF were detected by western blot analysis, while mRNA expression of VEGF was analyzed by real time PCR. The results showed that garcinol not only downregulated the protein expression of VEGF, but also suppressed *VEGF* gene at transcription level in a time-dependent manner in HNSCC cells.

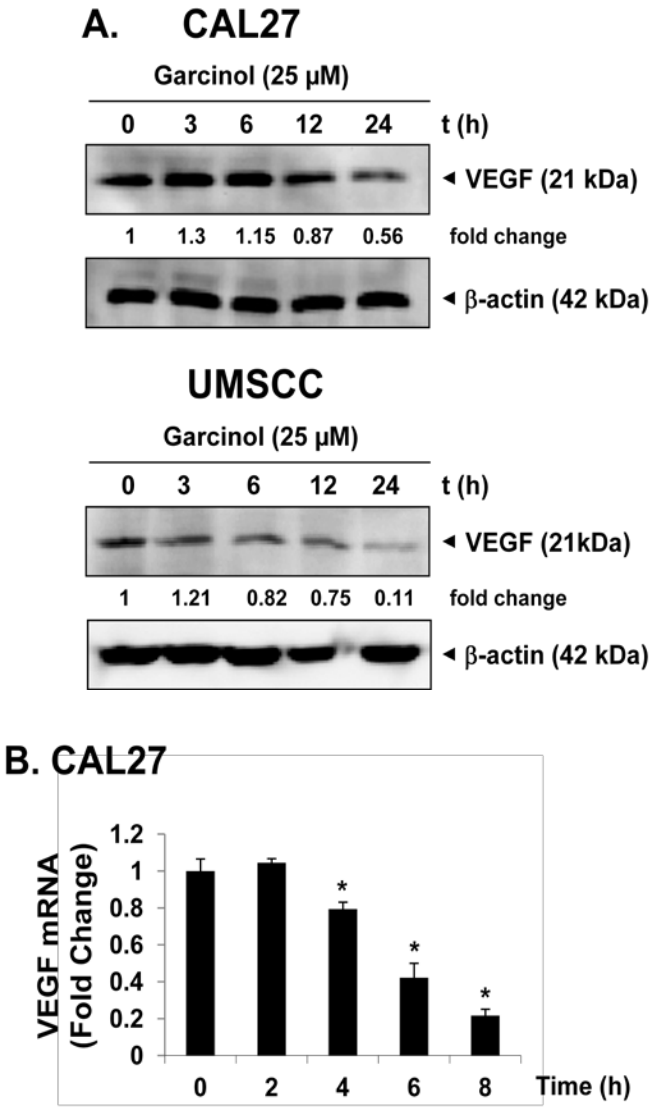


Figure 3.1.4. Garcinol downregulates the expression of VEGF in HNSCC cells.

A. CAL27 (*upper panel*) and UMSCC1 (*lower panel*) cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24h. Levels of VEGF in whole cell lysates were analyzed by western blot. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

B. CAL27 cells (1×10^6/mL) were exposed to 25 μM garcinol for 0, 2, 4, 6, and 8h. The cells were then harvested for RNA extraction, after which the relative expression of mRNA was evaluated by real time PCR. (*$p < 0.05$)

3.1.5 Garcinol significantly abrogates HNSCC cell migration and invasion.

The ability of cancer cells to undergo migration and invasion allow them to disseminate into the circulation, and promote metastatic progression into the distant organs (255, 256). To assess the anti-migratory and anti-invasive potential of garcinol, we examined its effects on the chemotactic motility of HNSCC cells using the wound-healing migration and invasion assays.

3.1.5.1 Garcinol suppresses CXCL12-induced HNSCC cell migration.

The wound-healing assay was performed using IBIDI μ-Dish which created a gap of 500 μm by the IBIDI culture insert. CAL27 cells seeded in the petri dish were treated with 15 μM garcinol for 8 h before exposed to chemokine CXCL12 (100 ng/mL) for 24 h, and the gap differences were compared at the time of 24 h incubation. We noticed that CXCL12 accelerated the "wound-healing" process, and the wound closing in cells treated with garcinol was much less as compared to the control.

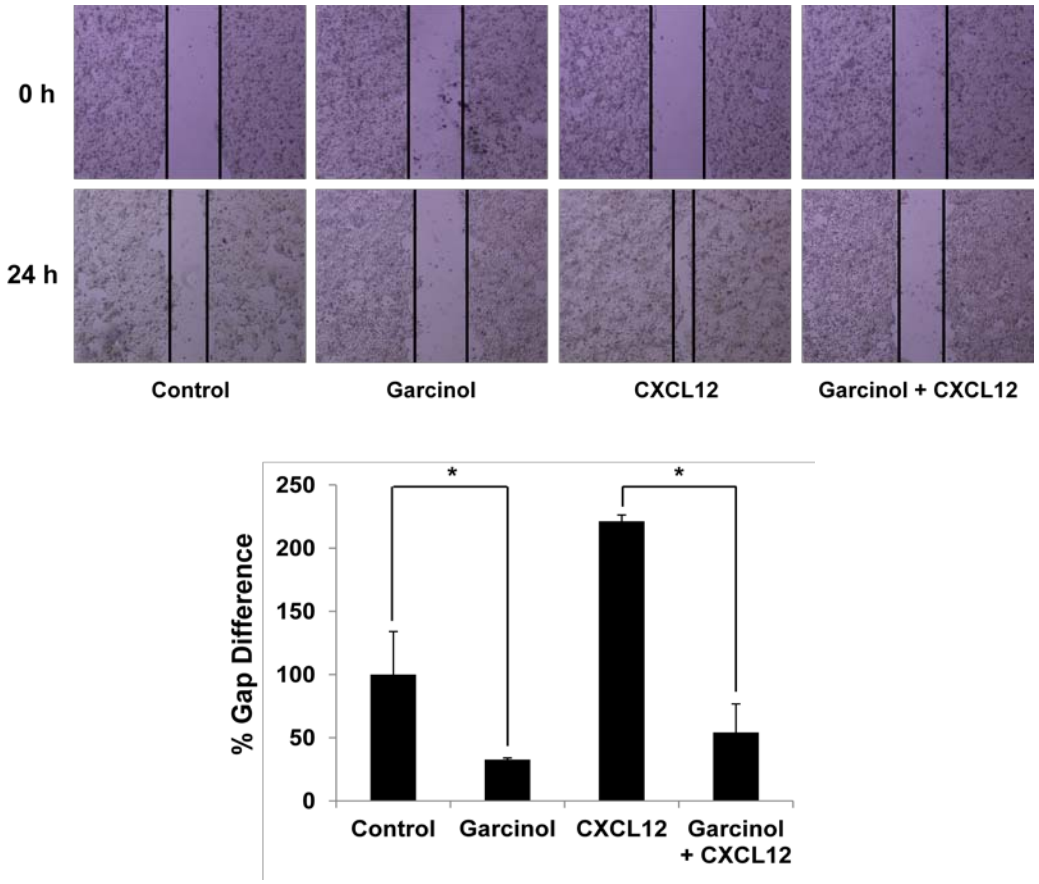


Figure 3.1.5.1. Garcinol significantly inhibits migratory ability of HNSCC cells.

Confluent monolayers of CAL27 cells with a 500 μ m gap created by IBIDI culture insert were treated with 15 μM garcinol in the presence or absence of CXCL12. *Upper panel*, representative images showed the same area at time zero and after 24 h of incubation. *Lower panel*, width of wound was measured at time zero and 24 h of incubation with and without garcinol treatment, and the graph was plotted against the percentage of migration distance the cells moved before and after treatment, normalized to control, *p <0.05.

3.1.5.2 Garcinol significantly blocks the invasive ability of HNSCC cells.

The invasive capability of HNSCCcells after garcinol treatment was examined using matrigel invasion assay. CAL27 cells plated in the matrigel chamber were pre-treated with 15 μM garcinol for 8 h, after which 100 ng/mL CXCL12 was added into the lower chamber of the 24-well plate for additional 24 h incubation. After the incubation, the cells invaded the matrigel to move towards the chemoattractant contacting outer layer. Invaded HNSCC cells were stained with crystal violet solution and analyzed under bright field microscopy. It was clearly evident from our *in vitro* invasion assay that garcinol inhibited the CXCL12-induced invasion of HNSCC cells.

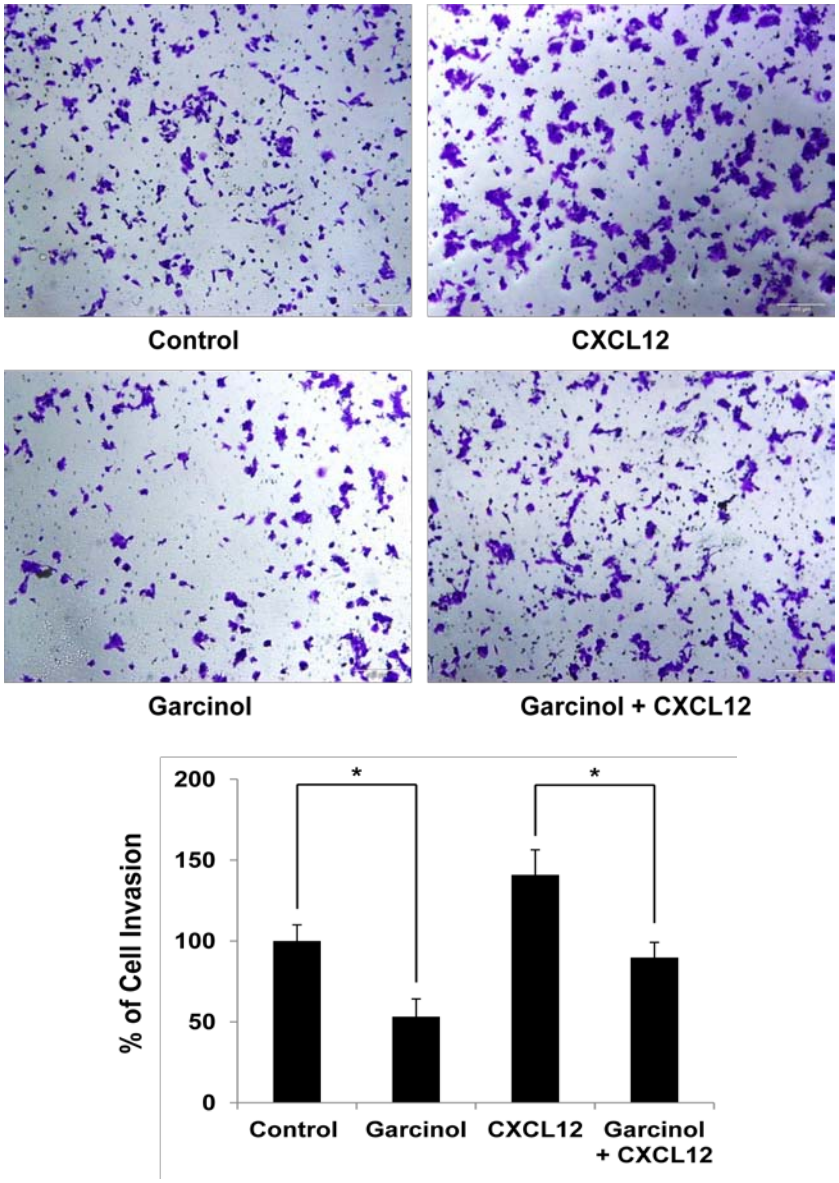


Figure 3.1.5.2. Garcinol inhibits invasive potential of HNSCC cells.

Upper panel, CAL27 cells (5×10^5 cells) were plated in the upper compartment of the transwell chamber, and then were treated with 15 μM garcinol in the presence or absence of CXCL12 (100 ng/mL). The chambers were then subjected to crystal violet staining to assess for cellular invasion. Representative images of at least two independent experiments are shown. *Lower panel*, the number of the invaded HNSCC cells with treatment was normalized against the untreated group, *p <0.05.

3.1.5.3 Garcinol donwnregulates the expression of proteins involved in HNSCC cell migration and invasion.

Western blot was performed to explore the effects of garcinol on key proteins regulating cell adhesion, migration and invasion processes. CAL27 cells were exposed to 25 μM garcinol for various time points as indicated, and whole cell lysates were analyzed for the expression of proteins of interest. We found that garcinol treatment indeed downregulated the expression of MMP-9, MMP-2, ICAM-1, and CXCR4 in a time-dependent manner in HNSCC cells.

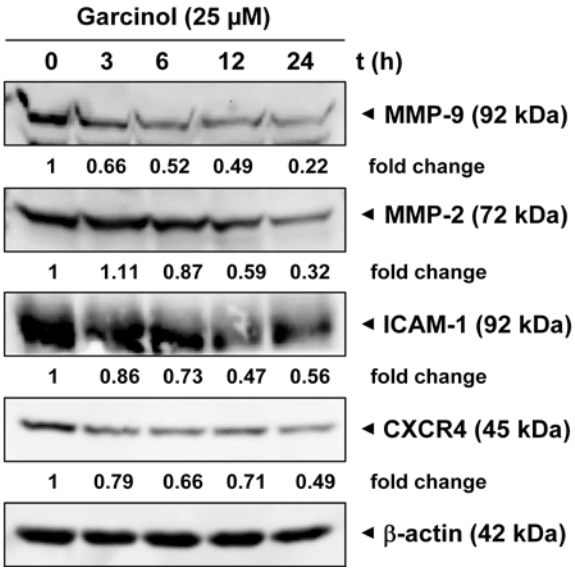


Figure 3.1.5.3. Garcinol donwnregulates the expression of proteins involved in HNSCC cell migration and invasion.

CAL27 cells (5×10^5/mL) were exposed to 25 μM garcinol for 0, 3, 6, 12, and 24 h. Levels of MMP-9, MMP-2, ICAM-1, and CXCR4 in whole cell lysates were analyzed by western blot. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

3.2 Effects of garcinol on STAT3 signaling cascade in HNSCC cells

3.2.1 HNSCC cells express constitutively active STAT3 protein.

To analyze the STAT3 activation profile in human HNSCC, panel of HNSCC cell lines were screened by western blot to detect the expression of phospho-STAT3 (p-STAT3) and the basal level of total STAT3. (Hereafter in this thesis, p-STAT3 indicates tyrosine phosphorylation of STAT3 at 705 residue unless stated otherwise). Whole cell extracts of CAL27, UMSCC1, MDA686LN, MDA686Tu and Tu138, and were prepared and used for the detection of p-STAT3 (Tyr-705) and total STAT3 expression. We found that majority of the HNSCC cell lines including CAL27, UMSCC1, Tu138 and MDA686Tu expressed substantial level of constitutively phosphorylated STAT3, while MDA686LN presented low expression level of phosphorylated STAT3.

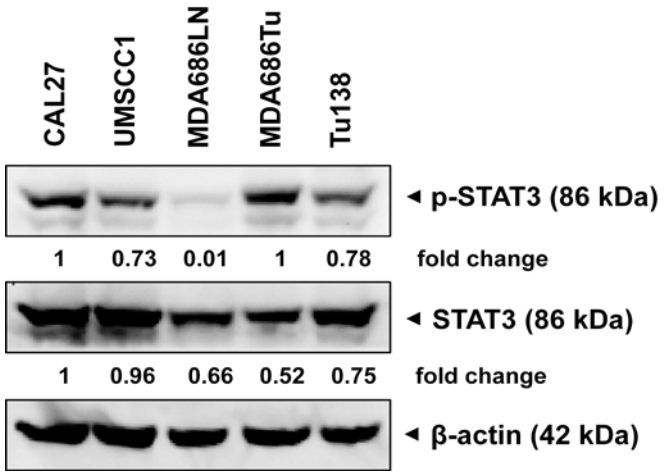


Figure 3.2.1. Basal level of p-STAT3 and STAT3 expression in HNSCC cell lines.

Whole cell lysates of different human HNSCC cell lines CAL27, UMSCC1, MDA686LN, MDA686Tu and Tu138 were extracted and subjected to western blot using antibodies against p-STAT3 and total STAT3. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots of at least two independent experiments have been shown.

3.2.2 IL-6 induces STAT3 phosphorylation in HNSCC cells.

The phosphorylation of STAT3 is known to be induced by various cytokines and growth factors such as IL-6 (70). We then tested if IL-6 stimulation could induce the phosphorylation of STAT3 in the HNSCC cell lines which do not display constitutive activated STAT3. To confirm this, one set of MDA686Tu cells were treated with fixed concentration of IL-6 for indicated time intervals, while the other set of cells were treated with different concentration of IL-6 for one specific time point. The STAT3 phosphorylation levels were then detected by western blot. It is clear from our results that IL-6 treatment induced substantial phosphorylation of STAT3 in a dose- and time-dependent manner.

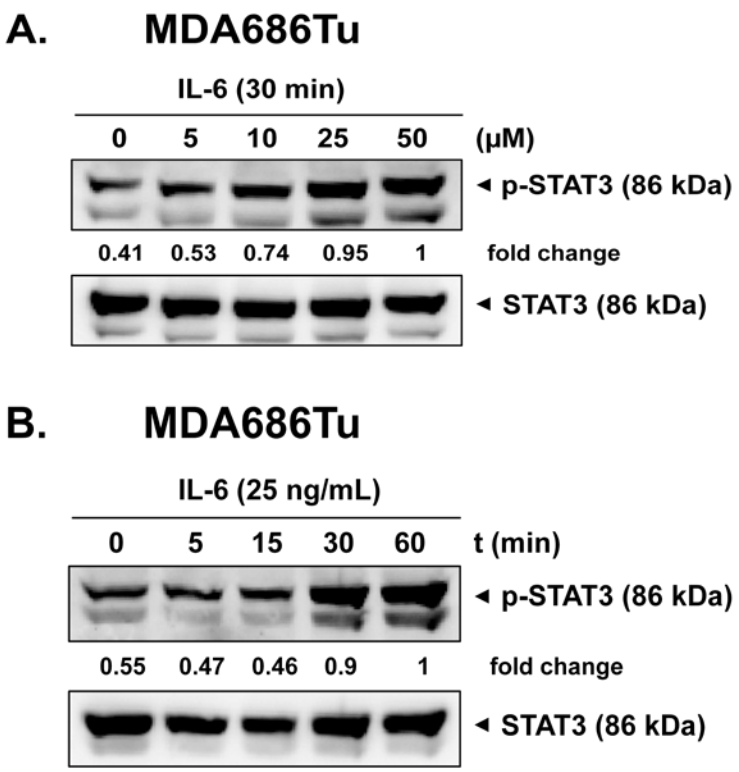


Figure 3.2.2. IL-6 stimulates STAT3 activation in HNSCC cells.

A. Western blot analysis of p-STAT3 in MDA686Tu cells (5×10^5/mL) treated with 0, 5, 10, 25, and 50 ng/mL of IL-6 for 30 min. STAT3 was detected on the same membrane after stripping to verify equal loading of proteins. Data representative of at least two independent experiments is shown.

B. Western blot analysis of p-STAT3 in MDA686Tu cells (5×10^5/mL) treated treated with 25 ng/mL of IL-6 for 0, 15, 30, 60, and 120 min. STAT3 was detected on the same membrane after stripping to verify equal loading of proteins. Data representative of at least two independent experiments is shown.

3.2.3 Garcinol suppresses the constitutive STAT3 phosphorylation in HNSCC cells.

Constitutive activation of STAT3 is frequently encountered in HNSCC cells and STAT3 inhibition can be considered as an important therapeutic option for the patients (83). To determine whether garcinol modulates STAT3 activation, HNSCC cells were exposed to various concentrations of garcinol for different time intervals as indicated, and then the extracted whole cell lysates were subjected to western blot to detect the STAT3 phosphorylation. The results showed that garcinol downregulated the constitutive STAT3 phosphorylation in a dose- and time-dependent manner with minimal effect on total STAT3 levels.

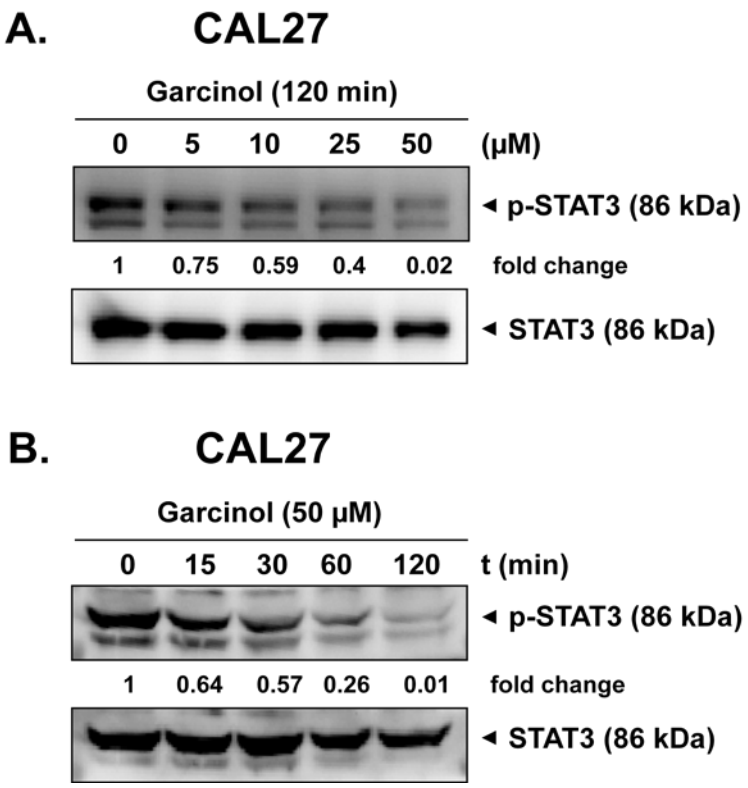


Figure 3.2.3. Garcinol suppresses the constitutive phosphorylation of STAT3 in HNSCC cells.

A. Western blot analysis of p-STAT3 in CAL27 cells (5×10^5/mL) treated with different concentrations of garcinol for 120 min. STAT3 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

B. Western blot analysis of p-STAT3 in CAL27 cells (5×10^5/mL) treated with 50 μM garcinol for 0, 15, 30, 60, and 120 min. STAT3 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

3.2.4 Garcinol does not affect serine phosphorylation of STAT3 in HNSCC cells.

Phosphorylation of a serine residue (serine 727) in the transcriptional activation domain of STAT3 enhances its transcriptional activity (257). Therefore, we examined whether garcinol could modulate the serine phosphorylation of STAT3 as well under the same experimental settings. Serine phosphorylation of STAT3 in CAL27 cells upon garcinol treatment was detected by western blot using antibody specific to STAT3 serine 727 residue. Interestingly, garcinol was found to have no significance influence on serine phosphorylation of STAT3, thereby indicating its specificity towards inhibition of p-STAT3 (Tyr 705) residue.

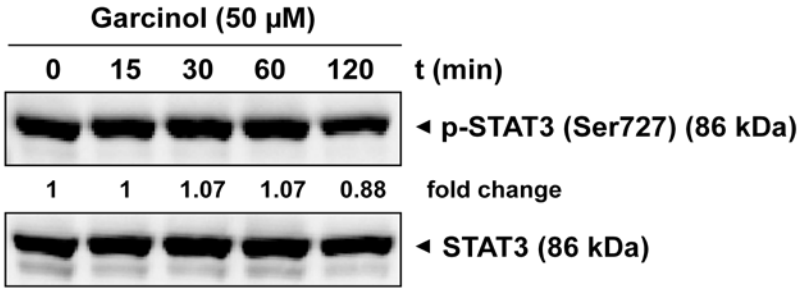


Figure 3.2.4. Garcinol did not affect serine phosphorylation of STAT3 in HNSCC cells.

Western blot analysis of p-STAT3 (Ser 727) in CAL27 cells (5×10^5/mL) treated with 50 μM garcinol for 0, 15, 30, 60, and 120 min. STAT3 was detected on the same membrane after stripping to verify equal loading of proteins. Data representative of at least two independent experiments is shown.

3.2.5 Garcinol abrogates IL-6-induced phosphorylation of STAT3 in HNSCC cells.

As observed in the above experiments, MDA686Tu exhibited elevated level of STAT3 phosphorylation upon IL-6 treatment. To analyze the potential inhibiting capability of garcinol on IL-6-induced STAT3 phosphorylation, western blot analysis was done. MDA686Tu cells were pretreated with 25 μM garcinol for 0, 1, 2, 4, and 6 h and then incubated with 25 ng/mL of IL-6 for 30 min. Thereafter, phosphorylation level of STAT3 was determined by western blot. Comparable to its inhibitory effects on constitutively active STAT3, garcinol also suppressed the IL-6-induced STAT3 phosphorylation in a time-dependent manner in MDA686Tu cells.

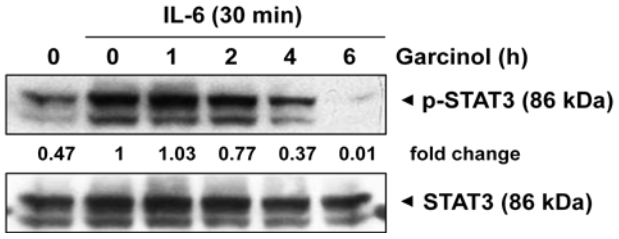


Figure 3.2.5. Garcinol inhibits IL-6-induced phosphorylation of STAT3 in HNSCC cells.

MDA686Tu cells (5×10^5/mL) were pretreated with 25 μM garcinol for different time points as indicated, and then incubated with 25 ng/mL of IL-6 for 30 min. Phosphorylation of STAT3 in whole cell lysates was assessed by western blot analysis using antibody against p-STAT3. STAT3 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

3.2.6 Garcinol depletes nuclear pool of STAT3 in HNSCC cells.

Following tyrosine phosphorylation and dimerization, STAT3 dimers translocate to the nucleus and regulate transcriptional activation (70). Since nuclear translocation is essential for the function of STAT3, we next investigated the effect of garcinol on nuclear translocation of STAT3. HNSCC cells were exposed to 50 μM garcinol for 120 min on chamber slides, after which the slides were analyzed for the intracellular distribution of STAT3 by immunocytochemistry. The obtained results showed that STAT3 localized in both cytoplasm and nuclei in vehicle control, while this protein predominantly resided in cytoplasm in the garcinol treated cells. Nuclear extracts from garcinol-treated HNSCC cells were further analyzed by western blot and showed a decrease in nuclear p65 protein level. Together with the immunocytochemistry study, our results suggest that garcinol blocked the nuclear translocation of STAT3 molecule into nucleus in CAL27 cells.

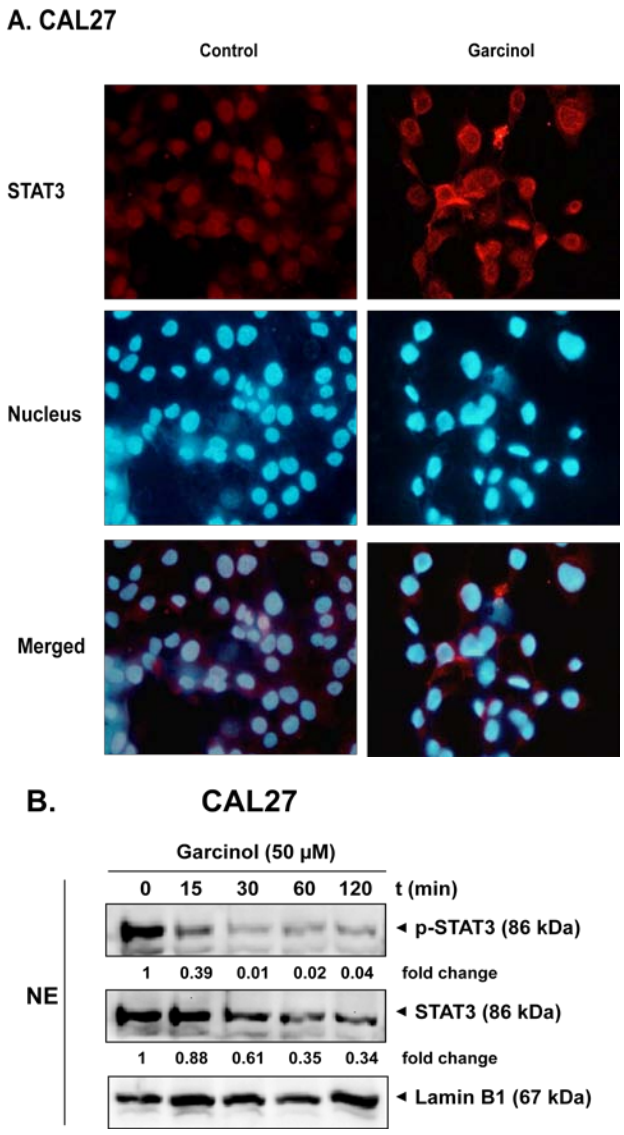


Figure 3.2.6. Garcinol inhibits the nuclear translocation of STAT3 in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed to 50 μM garcinol for 120 min, and then processed and analyzed by immunocytochemistry. Representative images of at least two independent experiments are shown.

B. The protein levels of p-STAT3 and total STAT3 in nuclear extracts from garcinol treated CAL27 cells were determined by western blot analysis. Lamin B1 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

3.2.7 Garcinol abrogates DNA-binding ability of STAT3 in HNSCC cells.

After translocating to the nucleus, STAT3 recognizes and binds to specific DNA elements to activate transcription (76), thus we aimed to determine whether garcinol inhibits the DNA-binding ability of STAT3. Nuclear proteins extracted from vehicle-and garcinol-treated CAL27 cells were analyzed by E LISA-based Trans AM™ STAT3 assay kit. Our result confirmed that garcinol significantly suppressed STAT3 DNA-binding activities in a time- and dose- dependent manner.

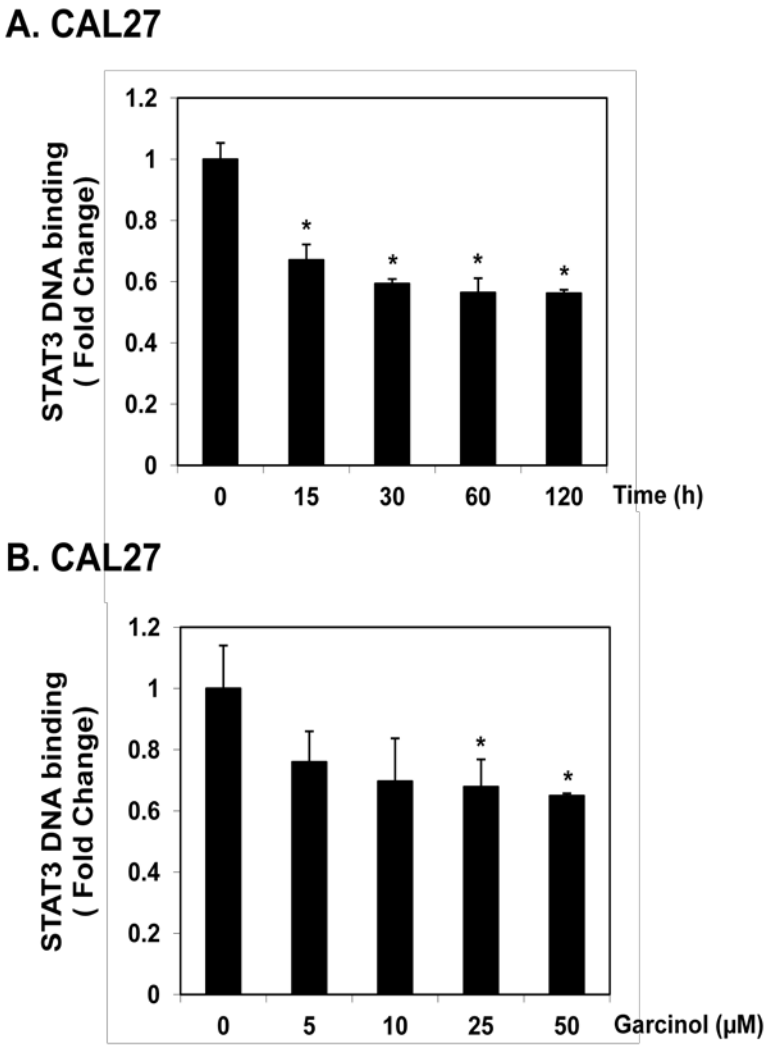


Figure 3.2.7. Garcinol abrogates DNA-binding ability of STAT3 in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed to 50 μM garcinol for 0, 15, 30, 60, and 120 min; nuclear extracts were analyzed by DNA-binding assay. (*p <0.05)

B. CAL27 cells (5×10^5/mL) were treated with 0, 5, 10, 25, a nd 50 μM garcinol for 120 min; nuclear extracts were analyzed by DNA-binding assay. (*p <0.05)

3.2.8 Garcinol blocks STAT3 dependent reporter gene expression in HNSCC cells.

Since garcinol has been found to inhibit the STAT3 DNA-binding activity as shown in the previous experiments, we next examined whether garcinol affects STAT3-dependent gene transcription. When the STAT3-luciferase (STAT3-Luc) plasmid transfected HNSCC cells were incubated with IL-6 or EGF, the luciferase activity was found to be increased; and the IL-6-induced or EGF-induced luciferase activities were suppressed in a dose-dependent manner in the presence of garcinol. These results indicated that garcinol abrogates STAT3-dependent reporter gene expression in HNSCC cells.

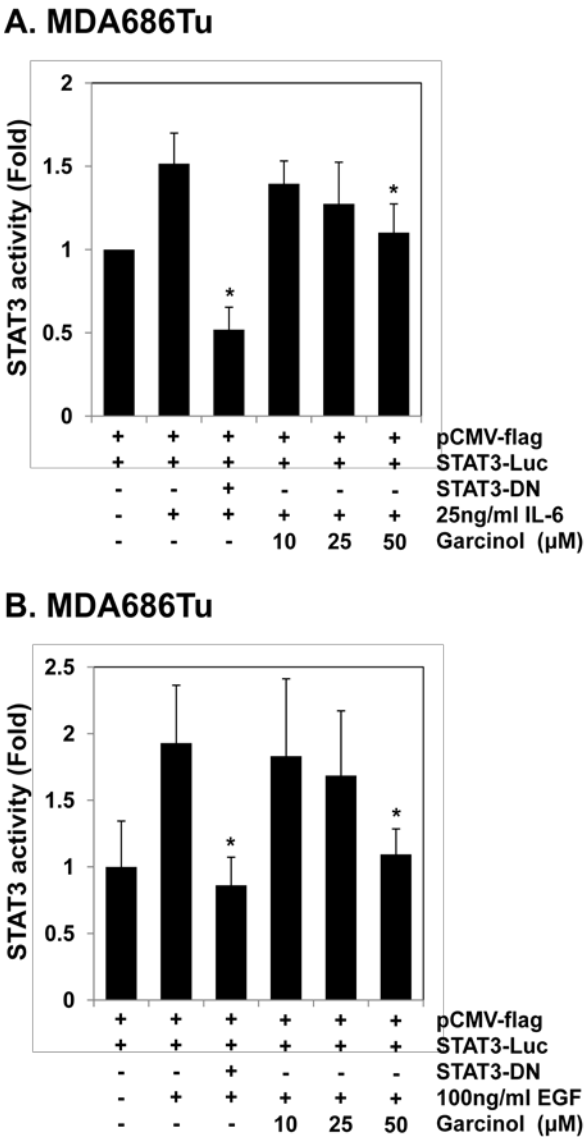


Figure 3.2.8. Garcinol inhibits IL-6 and EGF induced STAT3 dependent reporter gene expression.

A. Transiently transfected MDA686Tu cells (1×10^4/mL) were exposed to different concentrations of garcinol as indicated for 6 h, followed by addition of IL-6 (25 ng/mL) for 6 h. Luciferase activity was determined using Bright-Glo™ Luciferase Assay System. (*p <0.05)

B. Transiently transfected MDA686Tu cells (1×10^4/mL) were exposed to different concentrations of garcinol as indicated for 6 h, followed by addition of EGF (100 ng/mL) for 6 h. Luciferase activity was determined using Bright-Glo™ Luciferase Assay System. (*p <0.05)

3.2.9 Garcinol suppresses the constitutive activation of Src and JAK family kinases in HNSCC cells.

STAT3 activation is known to be regulated by Janus-activated kinase family and soluble tyrosine kinases Src (70). Thus, to determine whether the suppression of STAT3 activation upon garcinol treatment resulted from suppression of upstream kinases, we conducted western blot to examine the phosphorylation of Src, JAK1, and JAK2 in garcinol treated HNSCC cells. We noticed that garcinol inhibited the constitutive phosphorylation of Src in a time-dependent manner. Similarly, constitutively activated JAK1 and JAK2 were inhibited substantially by garcinol without affecting the total protein levels.

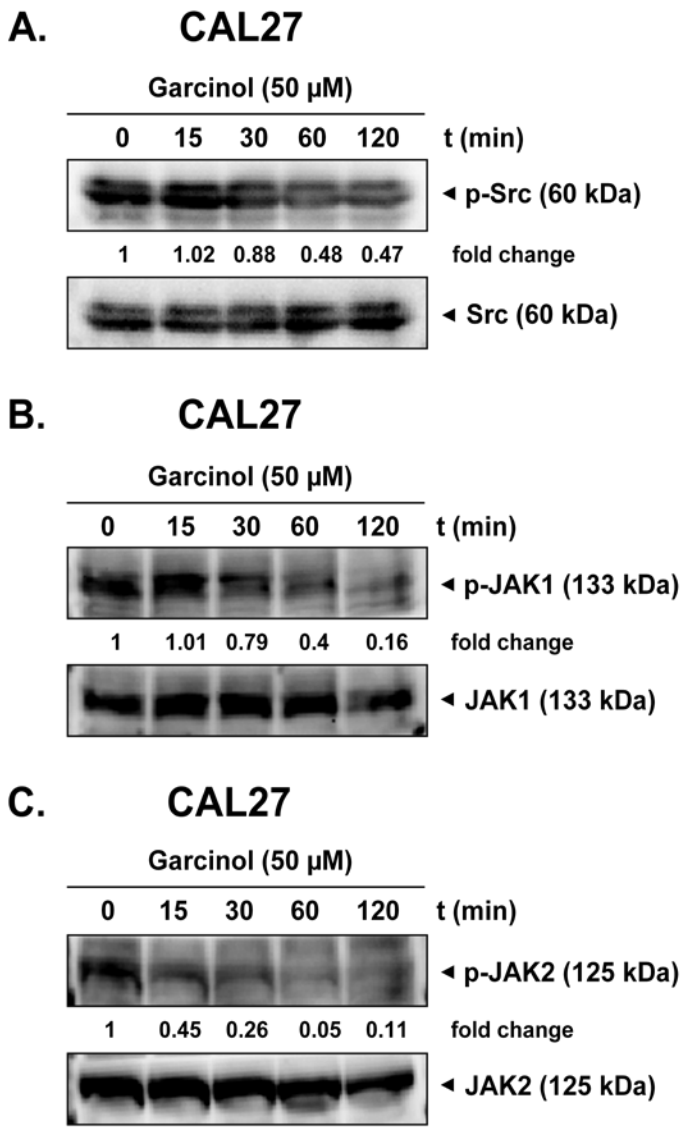


Figure 3.2.9. Garcinol suppresses the constitutive activation of Src and JAK family kinases in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed to 50 μM garcinol for 0, 15, 30, 60, and 120 min. Phosphorylation of Src in whole cell lysates was assessed by western blot using p-Src antibody. Src was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots of at least two independent experiments have been shown.

B. CAL27 cells (5×10^5/mL) were exposed to 50 μM garcinol for 0, 15, 30, 60, and 120 min. Phosphorylation of JAK1 in whole cell lysates was assessed by

western blot using p-JAK1 antibody. JAK1 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots of at least two independent experiments have been shown.

C. CAL27 cells (5×10^5/mL) were exposed to 50 μM garcinol for 0, 15, 30, 60, and 120 min. Phosphorylation of JAK2 in whole cell lysates was assessed by western blot using p-JAK2 antibody. JAK2 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots of at least two independent experiments have been shown.

3.2.10 Garcinol attenuates the IL-6-induced phosphorylation of Janus kinases in HNSCC cells.

The IL-6 stimulation leads to the activation of JAK family kinases which subsequently phosphorylate STAT3 (71). We next determined if garcinol inhibits STAT3 phosphorylation by modulating the activation of upstream JAK kinases. To analyze this aspect, MDA686Tu cells were first incubated with 25 μM garcinol for the indicated time points, after which the IL-6 treatment was applied. Our experimental findings indicated that IL-6 stimulated the phosphorylation of JAK1 and JAK2, and the IL-6-induced phosphorylation of both kinases was blocked upon garcinol treatment in a time-dependent manner.

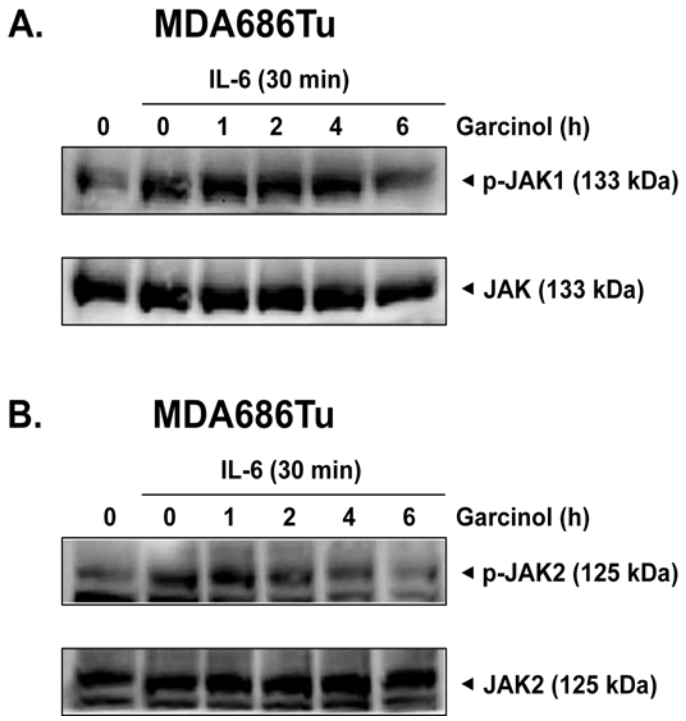


Figure 3.2.10. Garcinol inhibits IL-6-induced phosphorylation of JAK1 and JAK2 in HNSCC cells.

A.MDA686Tu cells (5×10^5/mL) were pretreated with 25 μM garcinol for 0, 1, 2, 4, and 6 h, and then incubated with 25 ng/mL of IL-6 for 30 min. Phosphorylation of JAK1 in whole cell lysates was assessed by western blot using antibody against p-JAK1. JAK1 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots of at least two independent experiments have been shown.

B.MDA686Tu cells (5×10^5/mL) were pretreated with 25 μM garcinol for 0, 1, 2, 4, and 6 h, and then incubated with 25 ng/mL of IL-6 for 30 min. Phosphorylation of JAK2 in whole cell lysates was assessed by western blot using antibody against p-JAK2. JAK2 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots of at least two independent experiments have been shown.

3.2.11 Tyrosine phosphatases may be involved in STAT3 inhibitory effects of garcinol in HNSCC cells.

Because STAT3 activation is also negatively regulated by several negative regulators such as protein tyrosine phosphatases (PTPs) (73, 258), we next examined whether the inhibition of STAT3 phosphorylation by garcinol could be due to the activation of PTPs. CAL27 cells were treated with the 50 μM garcinol and various concentrations of the broad spectrum tyrosine phosphatase inhibitor sodium pervanadate for 120 min, and the whole cell proteins were analyzed by western blot for the detection of p-STAT3. We found that sodium pervanadate reversed the garcinol-mediated inactivation of STAT3 in a dose-dependent manner.

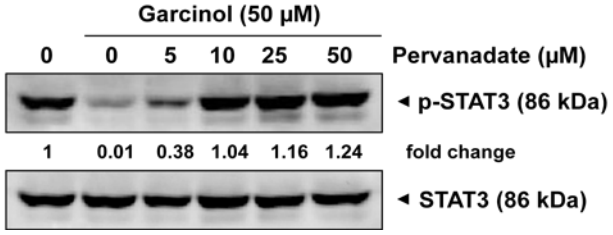


Figure 3.2.11. Sodium pervanadate blocked the inhibition of STAT3 phosphorylation induced by garcinol.

CAL27 cells (5×10^5/mL) were exposed to 0, 1, 5, 10, a nd 25 μ M sodium pervanadate and 50 μ M garcinol for 120 min. Phosphorylation of STAT3 in whole cell lysates was assessed by western blot using antibody against p-STAT3. STAT3 was detected on the same membrane after stripping to verify equal loading of proteins. Data representative of at least two independent experiments is shown.

3.2.12 Garcinol does not affect the activation/expression of protein tyrosine phosphatases (PTPs).

A number of PTPs have been implicated in the regulation of STAT3 signaling cascade (258). Thus, we examined whether garcinol modulate the cytoplasmic PTPs including Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP1), SHP2, PTP1B, and PTEN. CAL27 cells were exposed to 50 μM garcinol for various time intervals, and then western blot assay was done to detect the expression of SHP1, SHP2, PTP1B, and PTEN. The phosphorylation levels of SHP1 was also analyzed using phospho-specific SHP1 (S591and Y536) antibodies. Interestingly, we noted that garcinol treatment did not change the expression and/or phosphorylation levels of above tested PTPs in HNSCC cells.

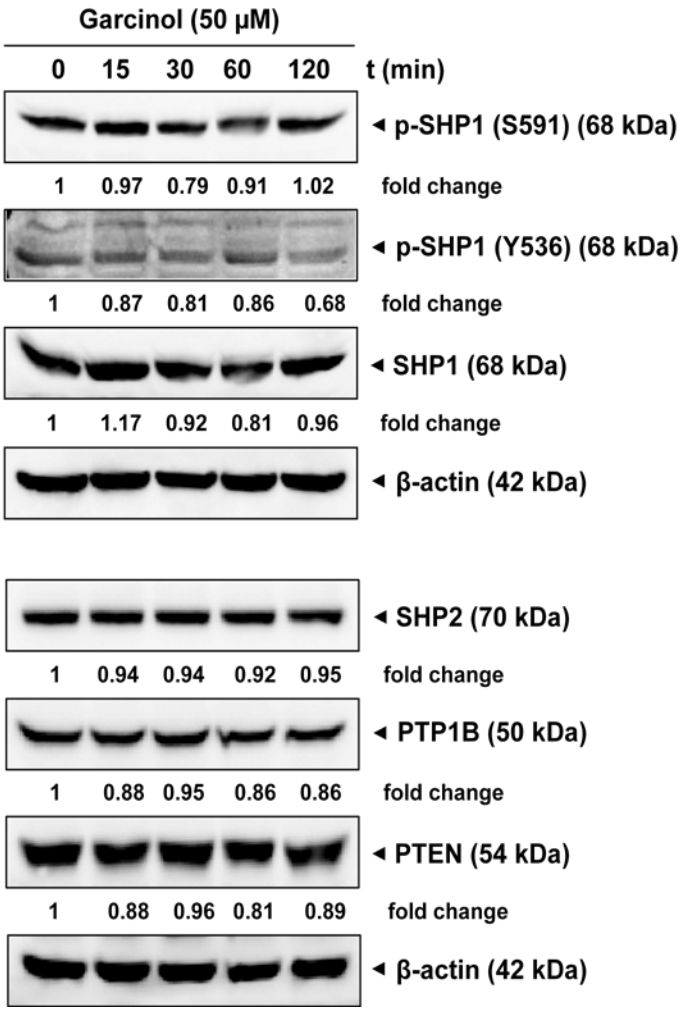


Figure 3.2.12. Garcinol does not affect the expression and/or activation of protein tyrosine phosphatases in HNSCC cells.

After incubation of 50 μM garcinol for 0, 15, 30, 60, a nd 120 min, the protein levels of SHP1, SHP2, PTP1B, PTEN, and p-SHP1 (S591and Y536) in whole cell lysates were determined by western blot. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots of at least two independent experiments have been shown.

3.3 Effects of garcinol on NF-κB signaling pathway in HNSCC cells

3.3.1 HNSCC cells express constitutively activated NF-κB.

Several prior studies have documented the prevalence of constitutive activated NF-κB in HNSCC cell lines and tumor tissues specimens (119-122). Thus, various HNSCC cell lines including CAL27, UMSCC1, MDA686LN, MDA686Tu, and Tu138 were examined for the NF-κB activation levels. Nuclear extracts of the above mentioned cell lines were prepared and analyzed by western blot for the expression of phospho-NF-κB (p-p65) and NF-κB (p65). We observed that CAL27 and UMSCC1 cell lines exhibit constitutively activated NF-κB levels, while the expression of active p65 in MDA686LN is moderate. On the contrary, MDA686Tu and Tu138 do not show substantial constitutively active NF-κB levels.

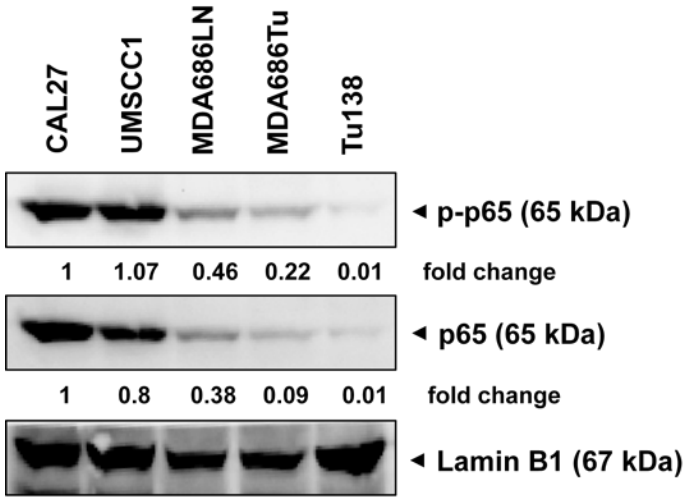


Figure 3.3.1. NF-κB (p65) activation profile in HNSCC cells.

Nuclear proteins of human HNSCC cells including CAL27, UMSCC1, MDA686LN, MDA686Tu, and Tu138 were extracted, and subjected to western blot using p-p65 and p65 specific antibodies. Lamin B1 was detected on the same membrane after stripping to verify equal loading of proteins. Representative pictures of at least two independent experiments have been shown.

3.3.2 Garcinol suppresses the constitutive NF-κB activation in HNSCC cells.

The constitutively activated NF-κB signaling in head and neck cancers has been reported to mediate resistance to apoptosis (124, 125); thus, we aimed to examine the effect of garcinol on the constitutive activation of NF-κB in HNSCC cells. HNSCC cells were exposed to fixed concentrations of garcinol for various time intervals as indicated. Nuclear lysates were extracted and subjected to western blot to detect p-p65 and p65 levels. It is evident from our experimental findings that garcinol suppressed the constitutive NF-κB phosphorylation in CAL27 in a time-dependent manner, and similar effects were observed in UMSCC1 cells with lower concentration of garcinol.

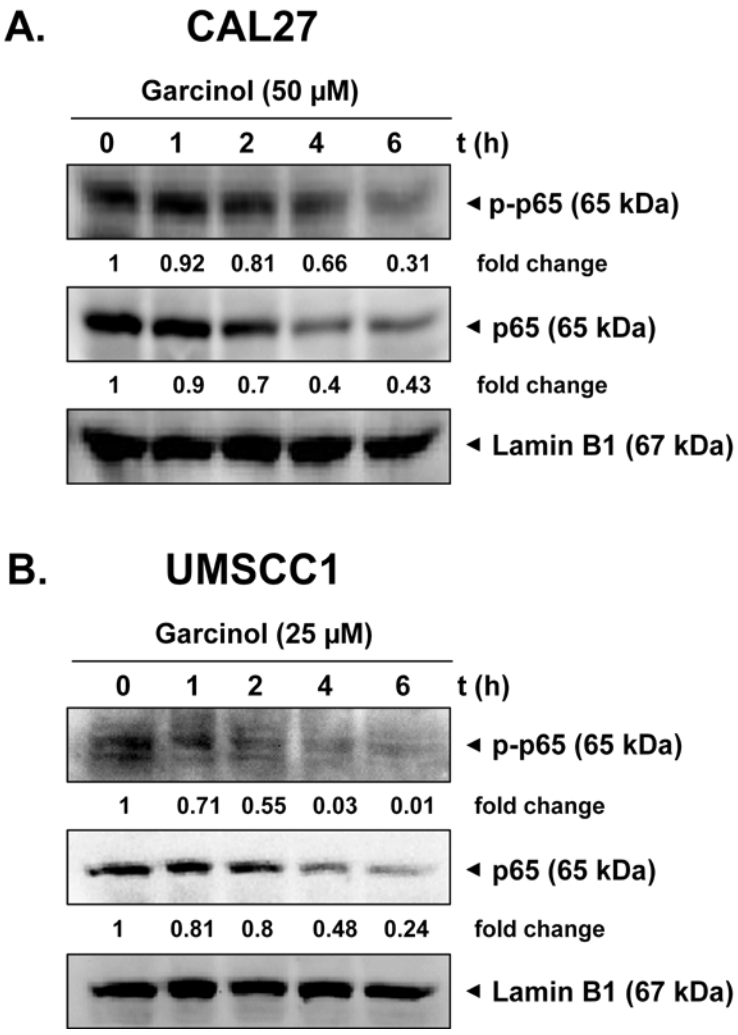


Figure 3.3.2. Garcinol suppresses the constitutive p65 phosphorylation and activation.

A. CAL27 cells (1×10^6/mL) were exposed to 50 μM garcinol for 0, 1, 2, 4, and 6 h, and then the nuclear protein levels of p-p65 and total p65 were detected by western blot. Lamin B1 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

B. UMSCC1 cells (1×10^6/mL) were exposed to 25 μM garcinol for 0, 1, 2, 4, and 6 h, and then the nuclear protein levels of p-p65 and total p65 were detected by western blot. Lamin B1 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

3.3.3 Garcinol abrogates phosphorylation and degradation of the constitutive IκBα in HNSCC cells.

As described in the Introduction section, NF-κB signaling is activated when NF-κB complex translocates to nucleus to regulate the transcription. The liberation of p65/p50 heterodimer requires the phosphorylation of IκBα which results in the proteasomal degradation of this NF-κB inhibitor (93). To study the involvement of this process in garcinol-induced inhibition of NF-κB activation, cytoplasmic fraction of garcinol treated HNSCC cells were extracted and analyzed for the p-IκBα and IκBα expression levels. We found that garcinol inhibited the phosphorylation of constitutive IκBα in a time-dependent manner, together with the increased level of total IκBα in both CAL27 and UMSCC1 cell lines.

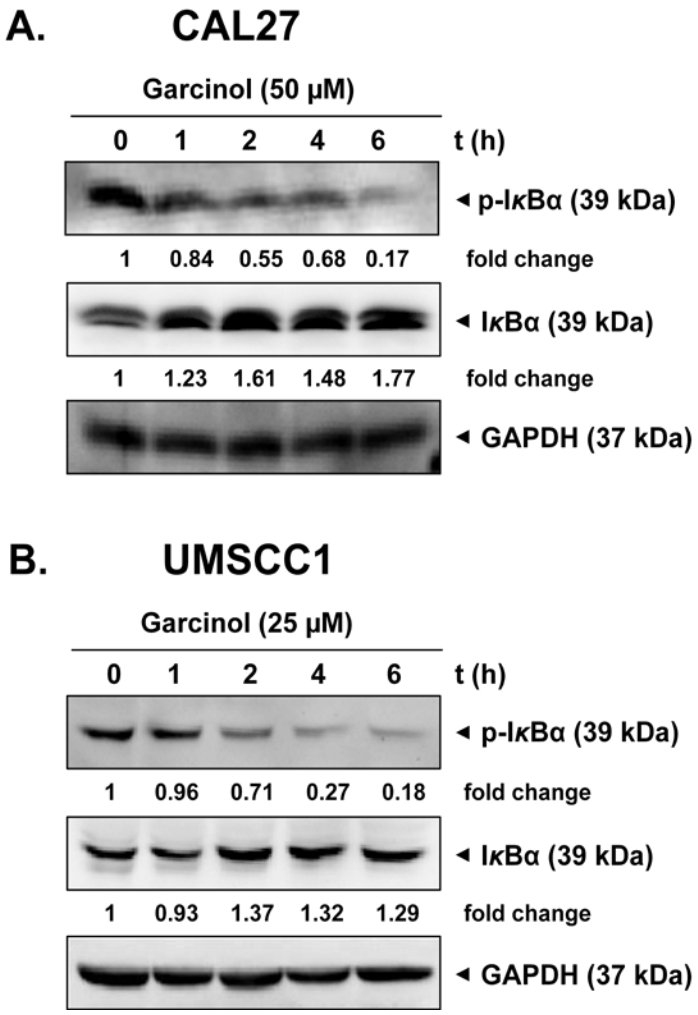


Figure 3.3.3. Garcinol inhibits the constitutive phosphorylation of IκBα in HNSCC cells.

A. CAL27 cells (1×10^6/mL) were exposed to 50 μM garcinol for 0, 1, 2, 4, and 6 h. Cytoplasmic proteins were prepared and subjected to western blot using p-IκBα and total IκBα antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control to verify equal loading of proteins was detected on the same membranes after stripping. Representative blots from at least two independent experiments have been shown.

B. UMSCC1 cells (1×10^6/mL) were exposed to 25 μM garcinol for 0, 1, 2, 4, and 6 h. Cytoplasmic proteins were prepared and subjected to western blot using p-IκBα and total IκBα antibodies. GAPDH as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

3.3.4 Garcinol reduces NF-κB DNA-binding activity in HNSCC cells.

To further evaluate the negative effect of garcinol on the constitutive NF-κB activation, garcinol-treated CAL27 and UMSCC1 cells were tested by ELISA-based DNA-binding assay. HNSCC cells were exposed for 6 h to the various concentrations of garcinol as indicated, and then collected for nuclear proteins extraction. Analysis of nuclear lysates extracted from both CAL27 and UMSCC1 cells using Trans AM™ NF-κB assay kit showed that garcinol significantly blocked NF-κB DNA-binding activities in a time-dependent manner.

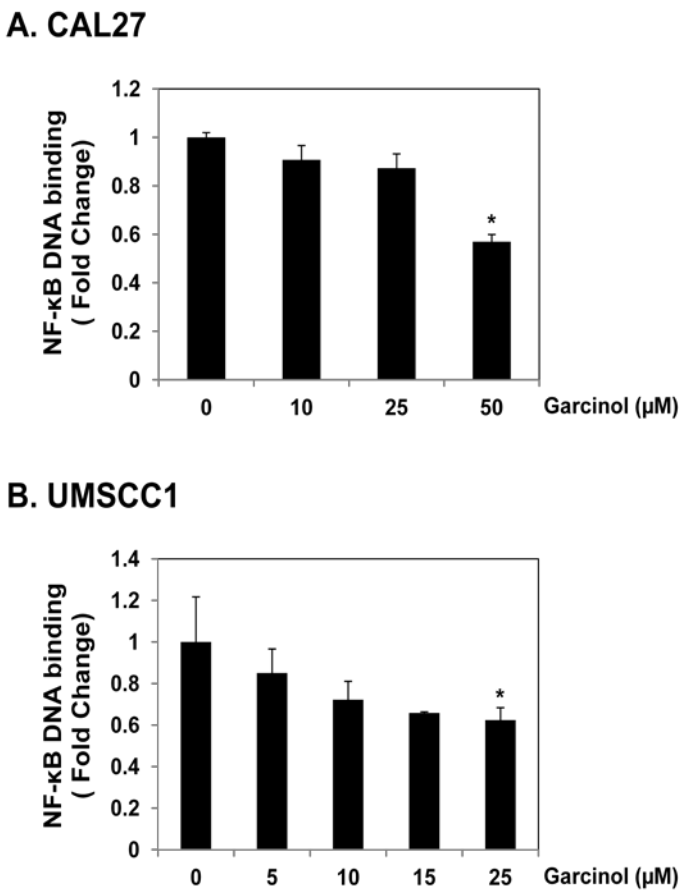


Figure 3.3.4. Garcinol inhibits NF-κB DNA-binding activity in HNSCC cells.

A. CAL27 cells (1×10^6/mL) were exposed for 6 h to 0, 10, 25, and 50 μM garcinol; nuclear extracts were analyzed by DNA-binding assay. (*p <0.05)

B. UMSCC1 cells (1×10^6/mL) were exposed for 6 h to 0, 5, 10, 15, and 25 μM garcinol; nuclear extracts were analyzed by DNA-binding assay. (*p <0.05)

3.3.5 Garcinol inhibits the constitutive activation of TAK1 and IKKα/β in HNSCC cells.

The degradation of negative regulator of NF-κB, IκBα protein, is modulated through IKK-dependent phosphorylation (93). We next determined if garcinol inhibits NF-κB by modulating the activation of upstream kinases TAK1 and IKK. Whole cell lysates were extracted from garcinol treated HNSCC cells, and subjected to western blot against p-TAK1, and p-IKKα/β specific antibodies. Our experimental results showed that garcinol substantially suppressed the phosphorylation of both TAK1 and IKK in a time-dependent manner.

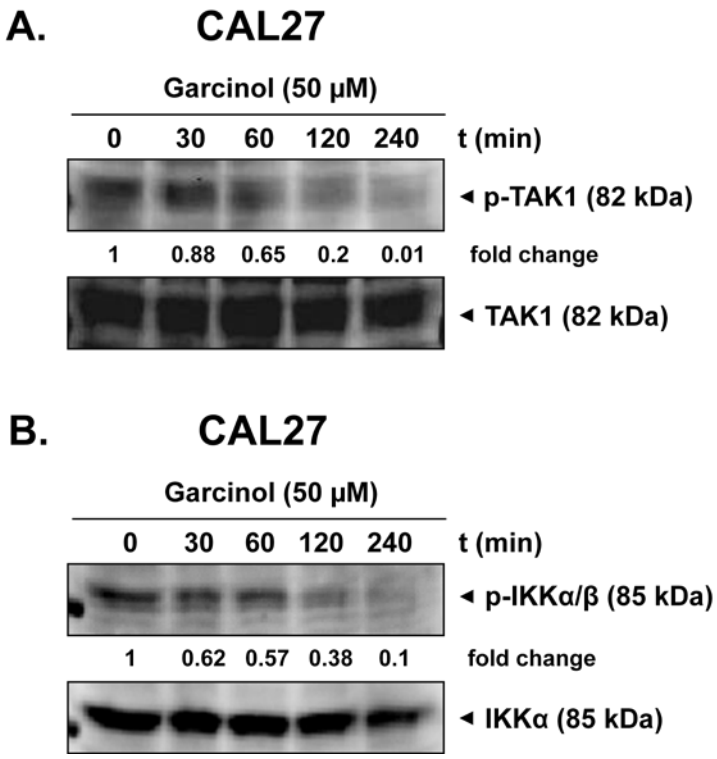


Figure 3.3.5. Garcinol inhibits the activation of upstream kinases involved in the NF-κB signaling in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed to 50 μM garcinol for 0, 30, 60, 120 , and 240 min; whole cell lysates were extracted and subjected to western blot using p-TAK1 antibody. TAK1 was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

B. CAL27 cells (5×10^5/mL) were exposed to 50 μM garcinol for 0, 30, 60, 120, and 240 m in; whole cell lysates were extracted and subjected to western blot using p-IKKα/β antibody. IKKα was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from of at least two independent experiments have been shown.

3.4 Potential effect of garcinol on the Akt signaling pathway in HNSCC cells

3.4.1 Garcinol suppresses constitutive Akt/mTOR/p70S6K activation in HNSCC cells.

Akt/mTOR/S6K1 is one of the major anti-apoptotic pathways that can confer the survival advantage and also mediate the resistance of HNSCC cells against various chemotherapeutic agents (259, 260). We therefore investigated whether garcinol can downregulate the constitutive Akt/mTOR/S6K1 activation in HNSCC cells. As shown in our results, constitutive activation of the serine/threonine protein kinase Akt was inhibited by garcinol in a time-dependent manner. In addition, the constitutive mTOR and S6K1 activation was also suppressed substantially upon garcinol treatment in HNSCC cells. These results indicate that garcinol exerts the anticancer effects through the negative regulation of multiple oncogenic molecules in HNSCC cells.

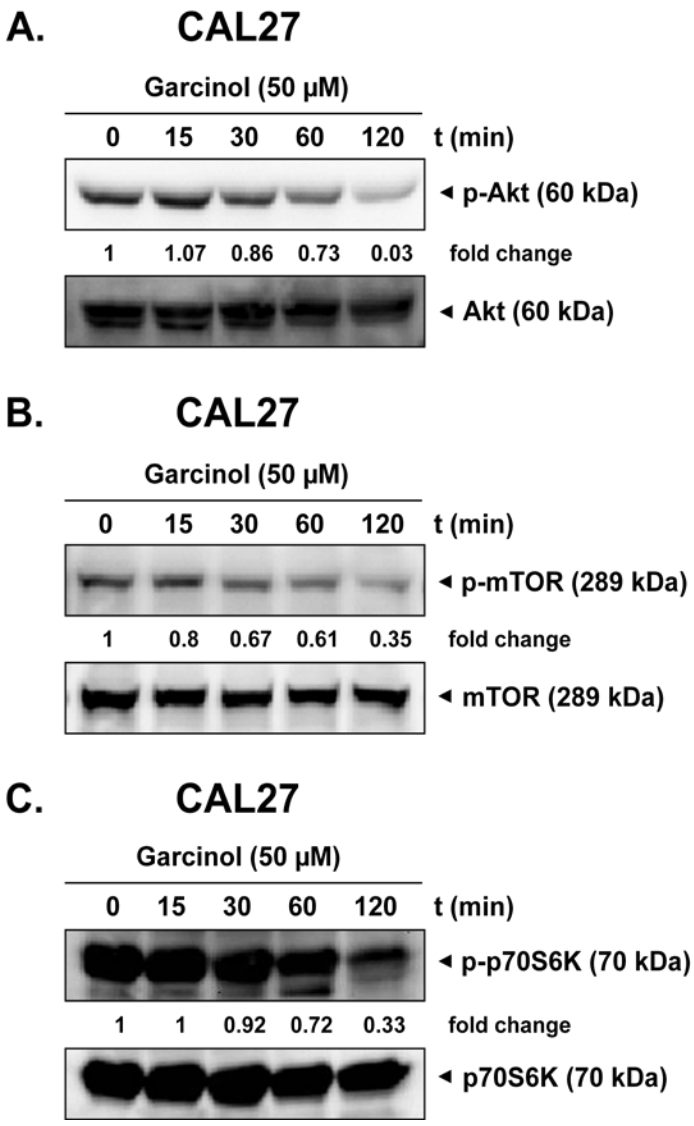


Figure 3.4.1. Garcinol inhibits Akt/mTOR/p70S6K axis in HNSCC cells.

A. Whole cell extracts from CAL27 cells (5×10^5/mL) treated 50 μM garcinol for 0, 15, 30, 60, and 120 min were analyzed by western blot using p-Akt antibody. Akt was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

B. Whole cell extracts from CAL27 cells (5×10^5/mL) treated 50 μM garcinol for 0, 15, 30, 60, and 120 min were analyzed by western blot using p-mTOR antibody. mTOR was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

C. Whole cell extracts from CAL27 cells (5×10^5/mL) treated 50 μM garcinol for 0, 15, 30, 60, a nd 120 m in were analyzed by western blot using p-p70S6K antibody. p70S6K was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

3.4.2 Garcinol abrogates the IL-6-induced phosphorylation of Akt in HNSCC cells.

Activated Akt has been shown to play a critical role in the mechanism of action of IL-6 (261). We also examined whether the effect of garcinol on IL-6-induced Akt activation. MDA686Tu cells were first incubated with 25 μM garcinol for the indicated time points, after which the IL-6 treatment was applied. Our experimental findings indicated that IL-6 stimulated the phosphorylation of Akt, and pretreatment with garcinol suppressed the Akt phosphorylation in a time-dependent manner.

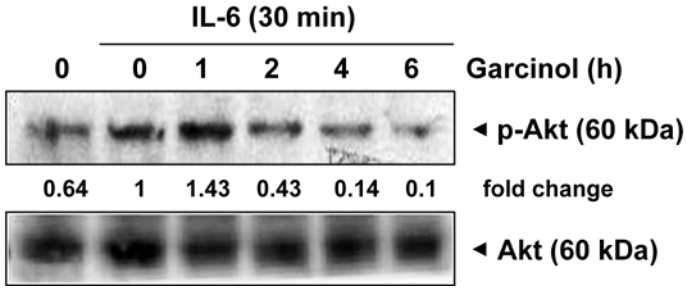


Figure 3.4.2. Garcinol inhibits IL-6-induced phosphorylation of AKT in HNSCC cells.

MDA686Tu cells (5×10^5/mL) were pretreated with 25 μM garcinol for 0, 1, 2, 4, and 6 h, and then incubated with 25 ng/mL of IL-6 for 30 min. Whole cell lysates were extracted and subjected to western blot using antibodies against p-AKT. AKT was detected on the same membrane after stripping to verify equal loading of proteins. Representative blots from at least two independent experiments have been shown.

3.5 Garcinol exerts significant growth inhibitory effects in HNSCC xenograft mouse model.

3.5.1 Pharmacokinetic properties of garcinol

In order to examine the efficacy of garcinol in a xenograft mouse model, a pharmacokinetic study of garcinol was conducted to define suitable doses for treatment of mice in pharmacodynamics study. Two doses of garcinol (0.5 and 2 mg/kg) were used for evaluation of pharmacokinetic property of garcinol through intraperitoneal administration. Blood was collected at 10 min, 30 min, 1, 2, 4, 6 and 8 h post dose, after which the processed serum samples were subjected to LC-MS/MS. After i.p. administration, garcinol was quickly absorbed into the bloodstream and reached a peak serum concentration (Cmax) of 1825.4 and 6635.7 nM at 0.5 h pos t dose for dose of 0.5 and 2 mg/kg, respectively. In addition, the area under the serum concentration-time curve (AUC) at 8 h is increased proportionally from 4298.3 to 16944.8 h*nM.

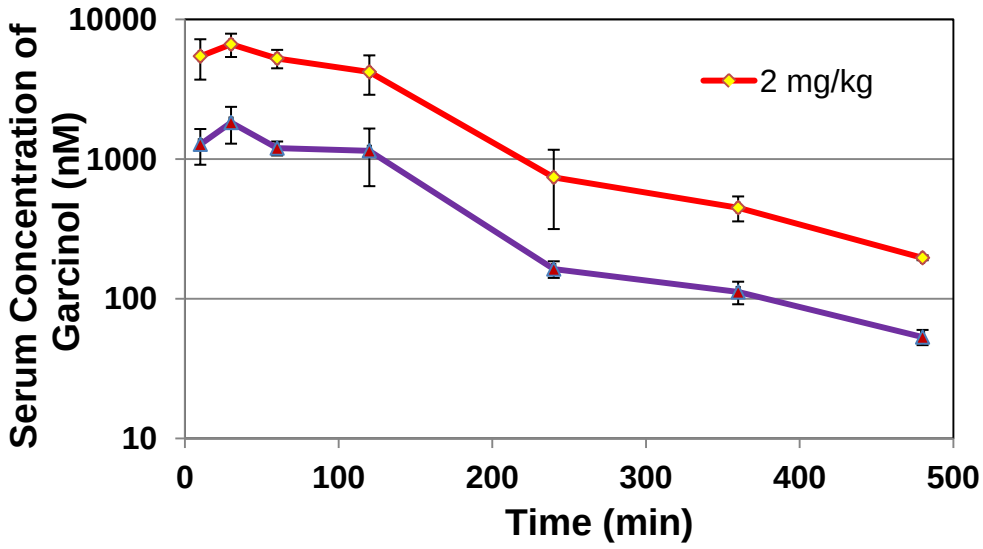


Figure 3.5.1. Pharmacokinetic studies with garcinol.

Athymic nu/nu female mice were dosed with 0.5 mg/kg and 2 mg/kg of garcinol, the serum concentrations of the drug were assessed at 10 min, 30 min, 1 h, 2 h, 4 h, 6 h, a nd 8 h pos t dose. Each point represents a m ean value of three serum concentrations. The error bars represent the mean ± SD.

3.5.2 Garcinol inhibits the growth of human HNSCC xenograft tumors.

Based on t he aforementioned results, we next analyzed whether garcinol suppresses the growth of HNSCC xenograft in athymic nu/nu mice. To establish the human HNSCC xenograft mouse model, CAL27 cells were subcutaneously injected in the right flank of athymic nu/nu male mice for the tumors to develop. The tumor bearing mice were then randomized into three groups and received control or garcinol treatment up to four weeks following the experimental protocol. Tumor diameters and body weight were measured twice a week. We noticed that the tumors in the control mice grew rapidly compared to garcinol treated groups. From the third week onwards, mice receiving garcinol treatment were observed to have lower tumor volumes compared with the control group, and the trend became more statistically significant as the treatment was continued.

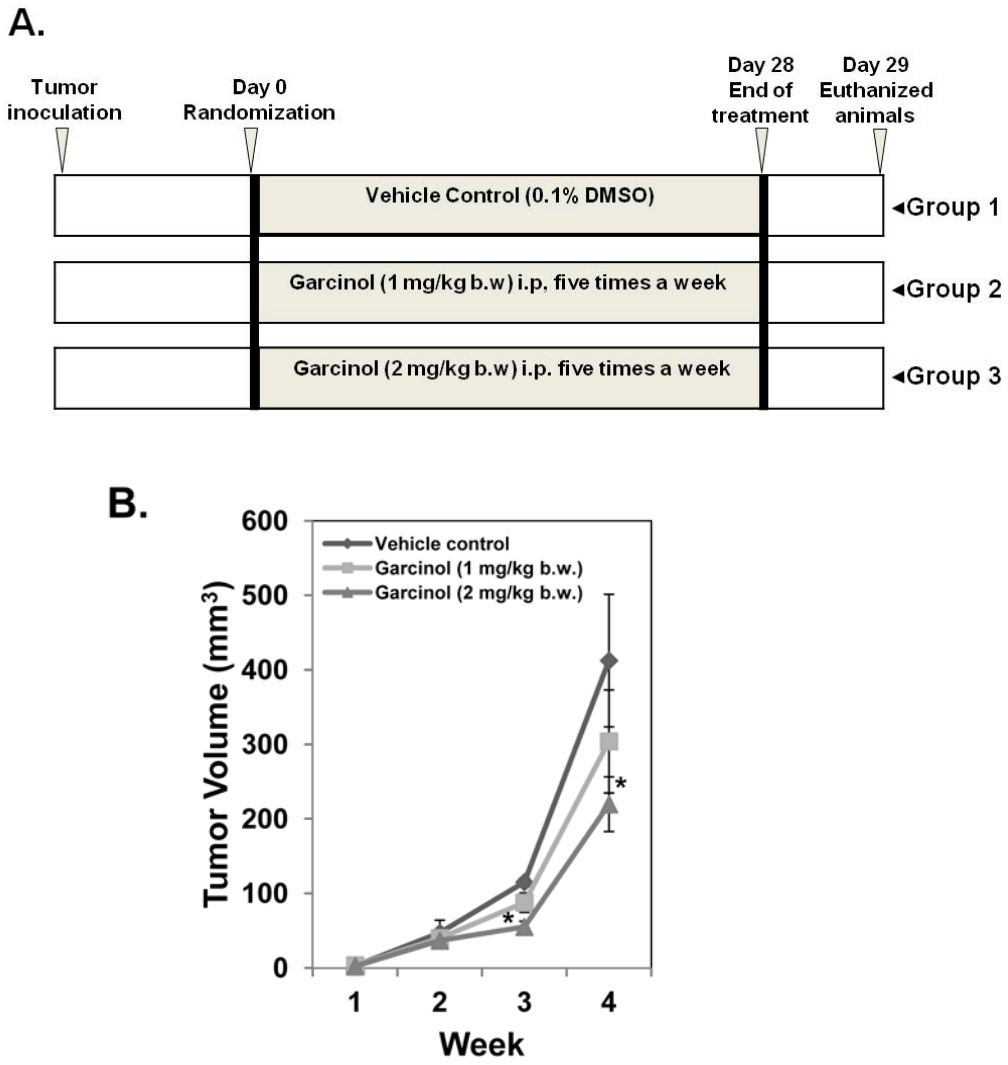


Figure 3.5.2. Garcinol inhibits the growth of human HNSCC xenograft tumors.

A. Schematic representation of experimental protocol described in "Materials and Methods". Group I was given 0.1% DMSO alone, group II was given garcinol (1 mg/kg body weight), and group III was given garcinol (2 mg/kg body weight).

B. The diameters of the tumor were measured twice per week, after which the tumor volume was calculated as: (longest diameter) × (shortest diameter)^2) × 0.5. (*p <0.05)

3.5.3 Garcinol inhibits the constitutive STAT3 and NF-κB activation in HNSCC tumor tissues.

Our data obtained from *in vitro* experiments has demonstrated that garcinol significantly inhibited the STAT3 and NF-κB activation in HNSCC cell lines. To determine whether garcinol also modulates these signaling cascades to elicit its anti-cancer effects *in vivo*, tumor tissues from xenograft mice were studied by immunohistochemistry analysis. At the end of the four week therapy, animals were sacrificed; thereafter the tumor tissues were excised from all the groups and processed by immunohistochemistry. We observed that garcinol significantly inhibited the constitutive STAT3 activation and p65 expression in garcinol-treated groups, comparing to the vehicle-treated control group.

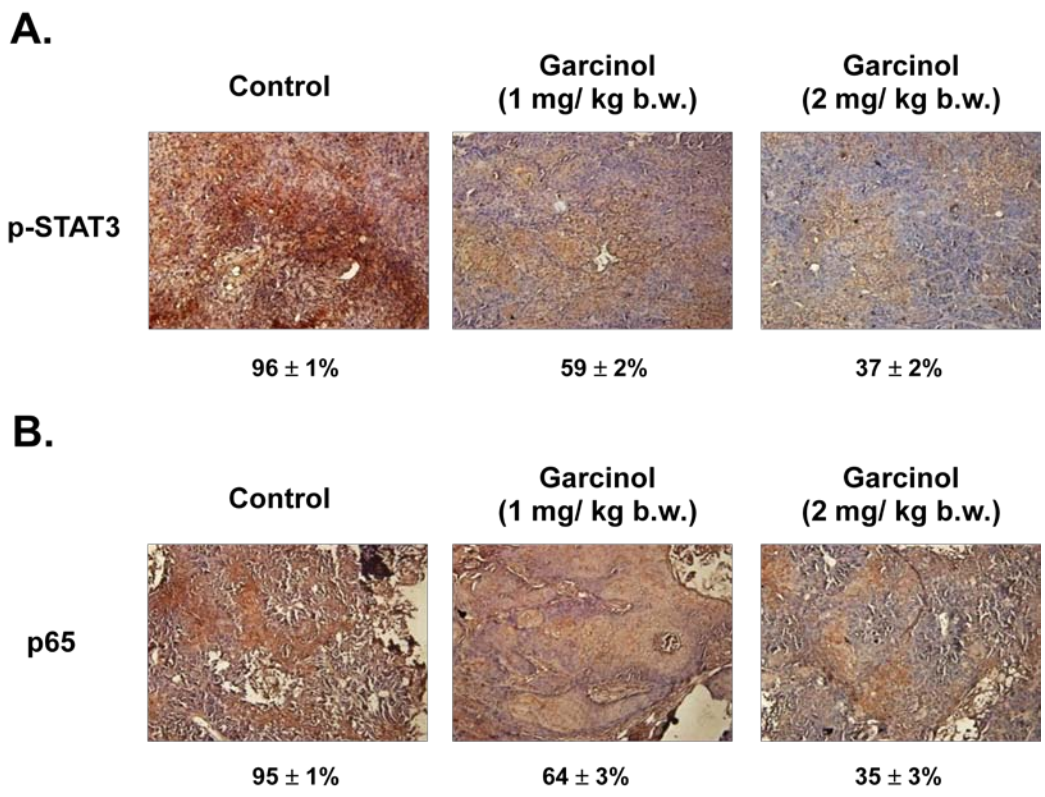


Figure 3.5.3. Garcinol donwregulates p-STAT3 and p65 expression *in vivo* in HNSCC xenograft mouse model.

A. Tumor tissues obtained were subjected to immunohistochemistry using p-STAT3 specific antibody. Representative image for each group is shown above.

B. Tumor tissues obtained were subjected to immunohistochemistry using p65 specific antibody. Representative image for each group is shown above.

3.5.4 Garcinol inhibits Ki-67 and CD31 expression in HNSCC tumor tissues.

Since Ki-67 is used as a cellular marker for proliferation (262) and CD31 index is a biomarker for microvessel density (263), we next determined to whether garcinol can modulate these biomarkers to manifest its observed anticancer effects against HNSCC. To explore this possibility, immunohistochemistry assay was performed using tumor tissues excised from the xenograft mice. Our experimental findings showed that garcinol downregulated the expression of both Ki-67 and CD31 in tumor tissues, suggesting it's anti-proliferative and anti-angiogenic potential *in vivo*.

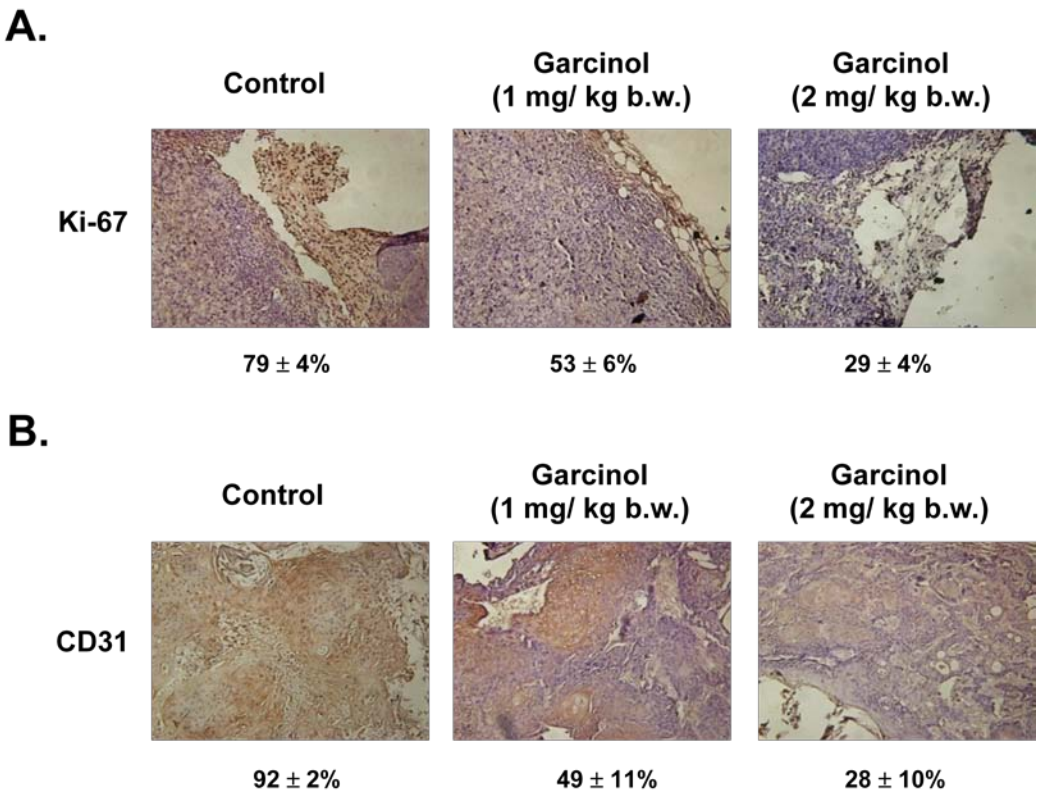


Figure 3.5.4. Garcinol inhibits Ki-67 and CD31 expression in HNSCC tumor tissues.

A. Tumor tissues obtained were subjected to immunohistochemistry using Ki-67 antibody. Representative image for each group is shown above.

B. Tumor tissues obtained were subjected to immunohistochemistry using CD31 antibody. Representative image for each group is shown above.

3.6 Garcinol sensitizes human HNSCC to cisplatin exposure in a xenograft mouse model.

Platinum compounds such as cisplatin and carboplatin are used as the first-line chemotherapy for the treatment of HNSCC (138, 139). However, they often lead to undesiable results due to the commonly encountered problems related to chemoresistance and toxicity (148, 150). Therefore, we also evaluated the potential of garcinol to sensitize HNSCC to cisplatin treatment both in cell lines and in a xenograft mouse model.

3.6.1 Garcinol suppresses the cisplatin-induced NF-κB activation in HNSCC cells.

3.6.1.1 Cisplatin induces NF-κB activation in HNSCC cells.

It has been reported that several chemotherapeutic agents including cisplatin induce the activation of NF-κB signaling to confer chemoresistance in several malignancies including HNSCC (115, 264, 265). To confirm these findings, MDA686LN cells were exposed to different concentrations of cisplatin, after which the nuclear proteins were extracted and analyzed by western blot and NF-κB DNA-binding assays. Interestingly, the western blot results showed that

cisplatin indeed induced the level of activated p65 in a dose- dependent manner. This observation was further confirmed by the elevated level of NF-κB DNA-binding activity in cisplatin treated cells.

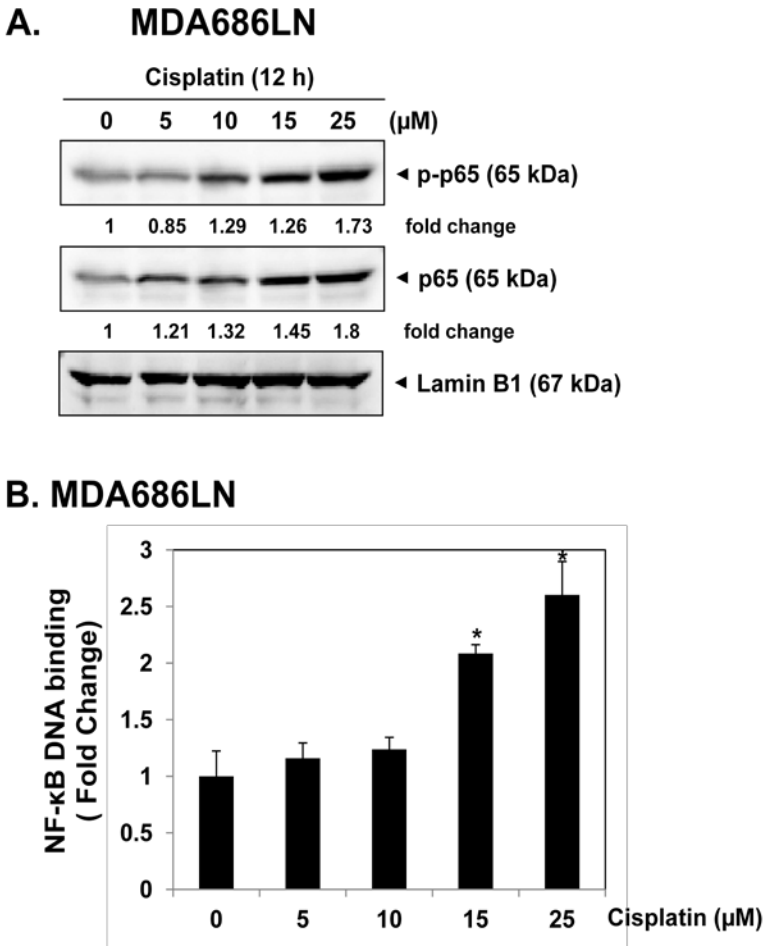


Figure 3.6.1.1. Cisplatin induces NF-κB activation.

A. MDA686LN cells (1×10^6/mL) were incubated with 0, 5, 10, 15, and 25 μM cisplatin for 12 h, and then the nuclear protein levels of p-p65 and total p65 were detected by western blot. Membrane was stripped and reprobed for lamin B1 to confirm equal loading. Data representative of at least two independent experiments is shown.

B. MDA686LN cells (1×10^6/mL) were incubated with 0, 5, 10, 15, and 25 μM cisplatin for 12 h, and nuclear lysates were extracted and subjected to NF-κB DNA-binding assay. (*$p < 0.05$)

3.6.1.2 Garcinol inhibits the cisplatin-induced NF-κB activation in HNSCC cells.

It is clearly evident from our earlier experiments that the therapeutic drug cisplatin was able to induce NF-κB activation in HNSCC. Also, we found that garcinol acted as a potent inhibitor of constitutive NF-κB activation in HNSCC cells. We next examined whether garcinol can also suppress the cisplatin-induced NF-κB activation. MDA686LN cells were exposed to various concentrations of garcinol and/or cisplatin as indicated for 12 h. The nuclear lysates were then prepared and subjected to western blot and NF-κB DNA-binding assays. Our results revealed that the cisplatin-induced NF-κB activation could also be significantly inhibited upon garcinol treatment in HNSCC cells.

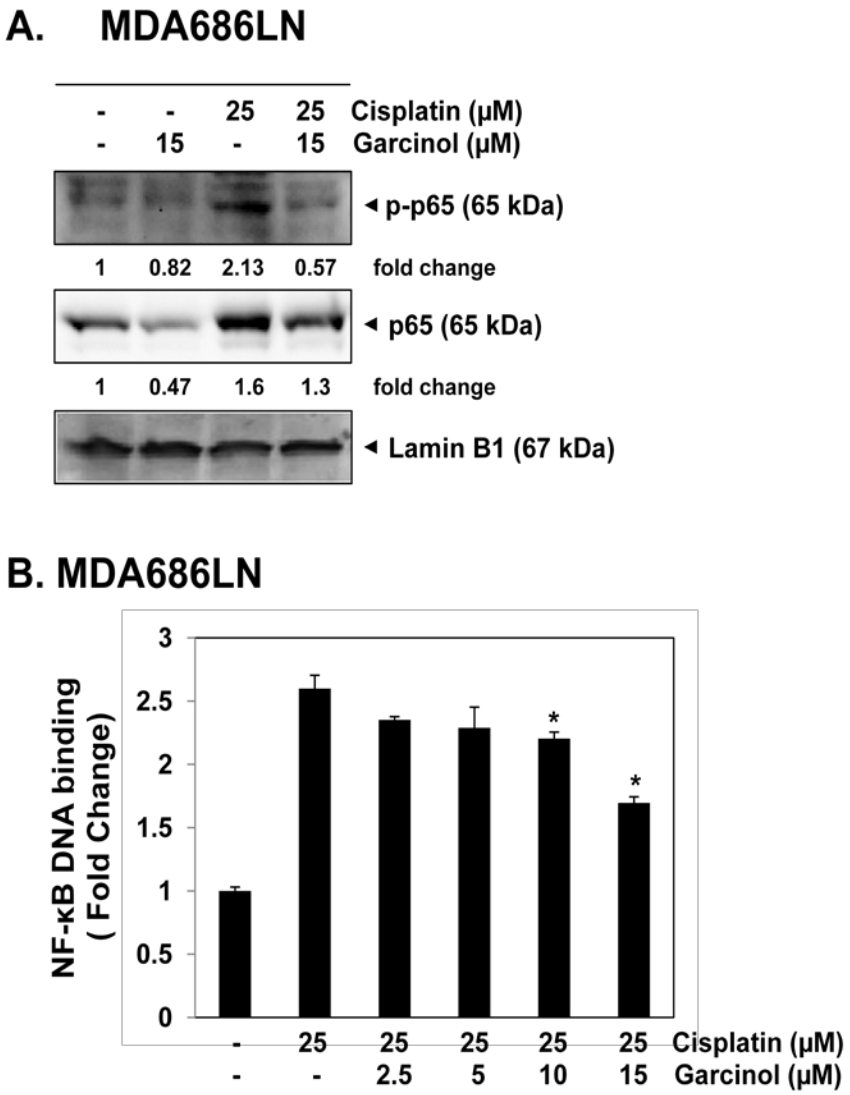


Figure 3.6.1.2. Garcinol inhibits cisplatin-induced NF-κB activation in HNSCC cells.

A. Nuclear lysates from solvent control, 15 μM garcinol, 25 μM cisplatin or their combination treated MDA686LN cells (1×10^6/mL) for 12 h were evaluated by western blot to assess the expression of p-p65 and p65 protein levels. Membrane was stripped and reprobed for lamin B1 to confirm equal loading. Representative blots from at least two independent experiments have been shown.

B. MDA686LN cells (1×10^6/mL) were exposed to various concentrations of garcinol and/or cisplatin as indicated for 12 h, and the nuclear proteins were extracted and subjected to NF-κB DNA-binding assay. (*$p < 0.05$)

3.6.1.3 Cisplatin induces the expression of various NF-κB-regulated gene products.

We have previously demonstrated that cisplatin was able to induce significant activation of NF-κB in HNSCC cells. To investigate the possible modulatory effect of cisplatin on NF-κB regulated gene products, we examined the expression level of various proteins by western blot analysis. MDA686LN cells were harvested at different time points after cisplatin exposure, after which whole cell lysates were extracted and subjected to western blot analyzed. We observed the expression of MMP-9, ICAM-1 and COX-2 were substantially increased after cisplatin treatment in a time-dependent manner in HNSCC cells.

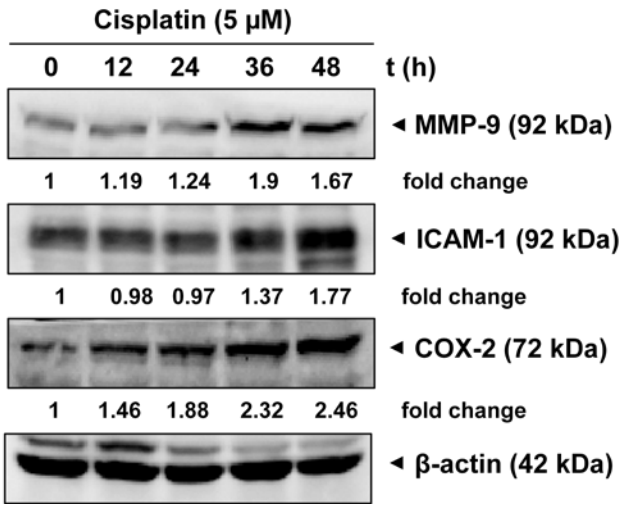


Figure 3.6.1.3. Cisplatin induces the expression of various NF-κB regulated gene products in HNSCC cells.

After incubation of 25 μM garcinol for 0, 12, 24, 36, and 48 h, the expression levels of MMP-9, ICAM-1, and COX-2 in whole cell lysates were determined by western blot. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

3.6.1.4 Garcinol downregulates the expression of NF-κB regulated proteins in HNSCC cells.

Given the inhibitory effect of garcinol on t he cisplatin-induced NF-κB activation demonstrated above, we next explored whether garcinol modulates the expression of NF-κB regulated proteins involved in HNSCC progression. MDA686LN cells were incubated with 15 μM garcinol, 5 μM cisplatin and their combination for 48 h; thereafter the whole cell lysates were extracted and subjected to western blot analysis. Our results revealed that garcinol treatment caused a substantial inhibition of both the constitutive and cisplatin-induced expression of MMP-9, ICAM-1 and COX-2 proteins in HNSCC cells.

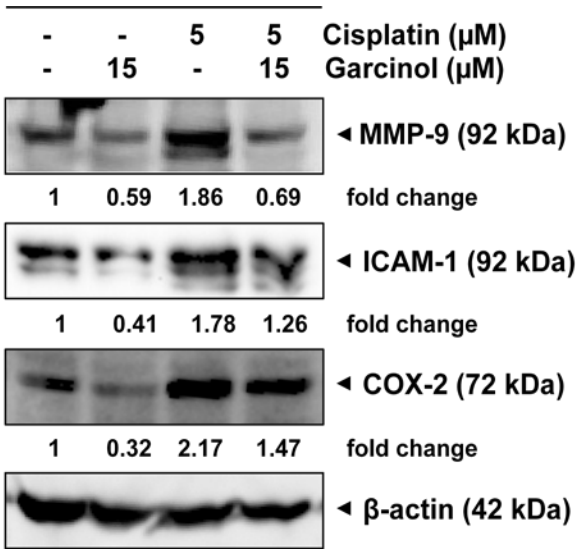


Figure 3.6.1.4. Garcinol downregulates the expression of NF-κB regulated proteins in HNSCC cells.

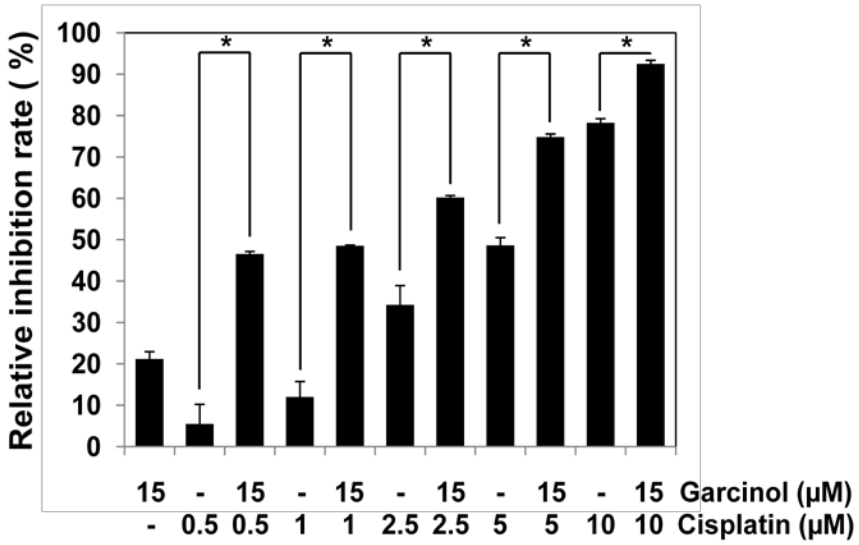
Whole cell lysates from solvent control, 15 μM garcinol, 5 μM cisplatin or their combination treated MDA686LN cells (5×10^5/mL) for 48 h were evaluated by western blot to assess the expression of MMP-9, ICAM-1, and COX-2 protein levels. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Representative blots from at least two independent experiments have been shown.

3.6.2 Garcinol potentiates the anti-cancer effects of cisplatin in HNSCC cells *in vitro.*

3.6.2.1 Garcinol enhances the cytotoxic effects of cisplatin in HNSCC cells *in vitro.*

Firstly, we examined whether garcinol treatment can augment the cytotoxic effect of cisplatin in HNSCC cells. CAL27 cells were treated with 15 μM garcinol and different concentrations of cisplatin alone and/or in combination for 48 h, after which the cell viability were analyzed by MTT assay. Our results showed that the combination of garcinol with cisplatin could produce enhanced growth inhibitory effect than either agent used alone. The combination effects were found to be synergistic, assessed by Chou-Talalay method using CompuSyn software.

A. CAL27



B. CAL27

Dose of garcinol (μM)	Dose of Cisplatin (μM)	CI Value
15.0	0.5	0.15995
15.0	1.0	0.26651
15.0	2.5	0.43118
15.0	5.0	0.51447
15.0	10.0	0.35462

Figure 3.6.2.1. Garcinol augments the cytotoxic effect of cisplatin in HNSCC cells.

A. CAL27 cells (5×10^5/mL) were exposed for 48 h to different concentrations of garcinol and cisplatin, and their combination as indicated above and then subjected to MTT assay to analyze the viability of tumor cells. Differences in cell growth after exposure to garcinol and cisplatin, alone and in combination, were determined with the one-way ANOVA test, * $p < 0.05$.

B. The synergy quantification of drug combination study was done using Chou-Talalay method. The degree of synergy was assessed based on the combination index (CI), where CI values of >1.0 implies antagonism, 1.0 implies additive and <1.0 implies synergistic effect relationships.

3.6.2.2 Garcinol augments the apoptotic effects of cisplatin in HNSCC cells *in vitro.*

We next investigated whether garcinol when employed at suboptimal concentration can potentiate the apoptotic effect of cisplatin in HNSCSS cells. CAL27, UMSSC1 and MDA686LN cells were treated with 15 μ M garcinol and/or 5 μM cisplatin for 48 h, and then analyzed by flow cytometry and western blot assays. It is clearly evident from our data that combination of garcinol and cisplatin produced significant apoptosis in all three cell lines, as evidenced by increase in sub-G1 population, while either agent alone only induced moderate apoptotic cell death. Furthermore, co-treatment of garcinol and cisplatin more potently activated caspase-3 and caused PARP cleavage, compared to single agents used individually. Overall, our results suggest that garcinol can indeed substantially potentiate the apoptotic effects of cisplatin against HNSCC cell lines.

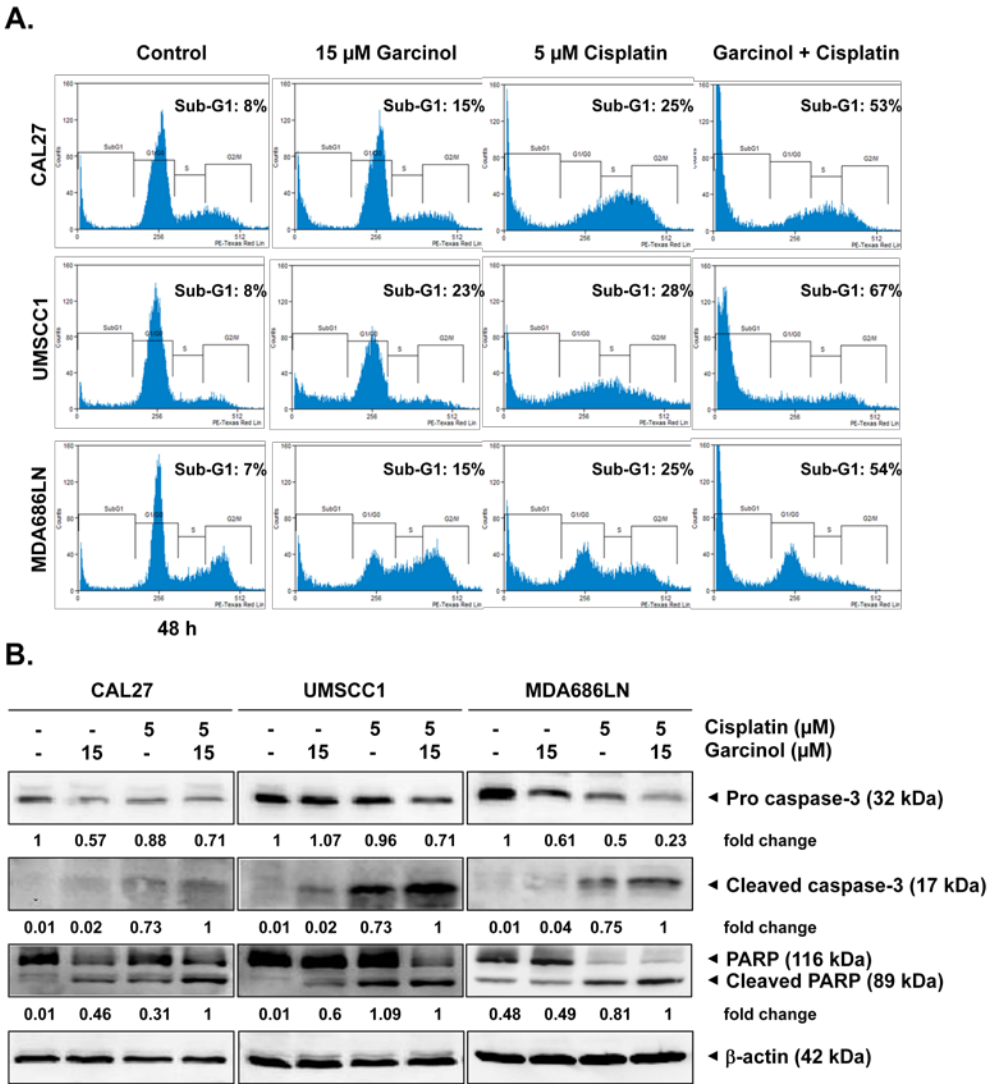


Figure 3.6.2.2. Garcinol augments the cisplatin-induced apoptosis in HNSCC cells.

A. CAL27, UMSSC1, and MDA686LN cells (5×10^5/mL) were exposed to 15 μM garcinol and/or 5 μM cisplatin for 48 h, after which the cells were collected for fixation, PI staining, and then subjected to FACS analysis. Representative graphs of at least two independent experiments are shown.

B. CAL27, UMSSC1 and MDA686LN cells (5×10^5/mL) were exposed to 15 μM garcinol and/or 5 μM cisplatin for 48 h; whole cell lysates were extracted and subjected to western blot using caspase-3 and PARP antibodies. β-actin as an internal control to verify equal loading of proteins was detected on the same membranes after stripping. Representative blots from at least two independent experiments have been shown.

3.6.3 Garcinol potentiates the anti-tumor effects of cisplatin in HNSCC xenograft mouse model.

3.6.3.1 Garcinol enhances the inhibition of tumor growth induced by cisplatin *in vivo.*

Based on the initial observation that garcinol has the capacity to enhance the apoptotic effects of cisplatin in HNSCC cell lines, we evaluated the *in vivo* therapeutic potential of garcinol and cisplatin either alone or in combination on the growth of HNSCC CAL27 xenografts in nude mouse. CAL27 cells were implanted subcutaneously into the right flank of athymic nude mice. The mice were then divided into four groups, one week post tumor inoculation when tumors reached 0.25 cm in diameter as per the study protocol. The efficacy of the treatment was evaluated by tumor volume during the four week treatment. Significant decreases in the tumor volume in single agent treated group were observed from week 2 until the end of the experiment, and the combined treatment exerted more pronounced effect. The tumor volume in the combination of garcinol and cisplatin was significant lower than garcinol alone group ($p < 0.05$) or cisplatin alone group ($p < 0.05$) on week 4.

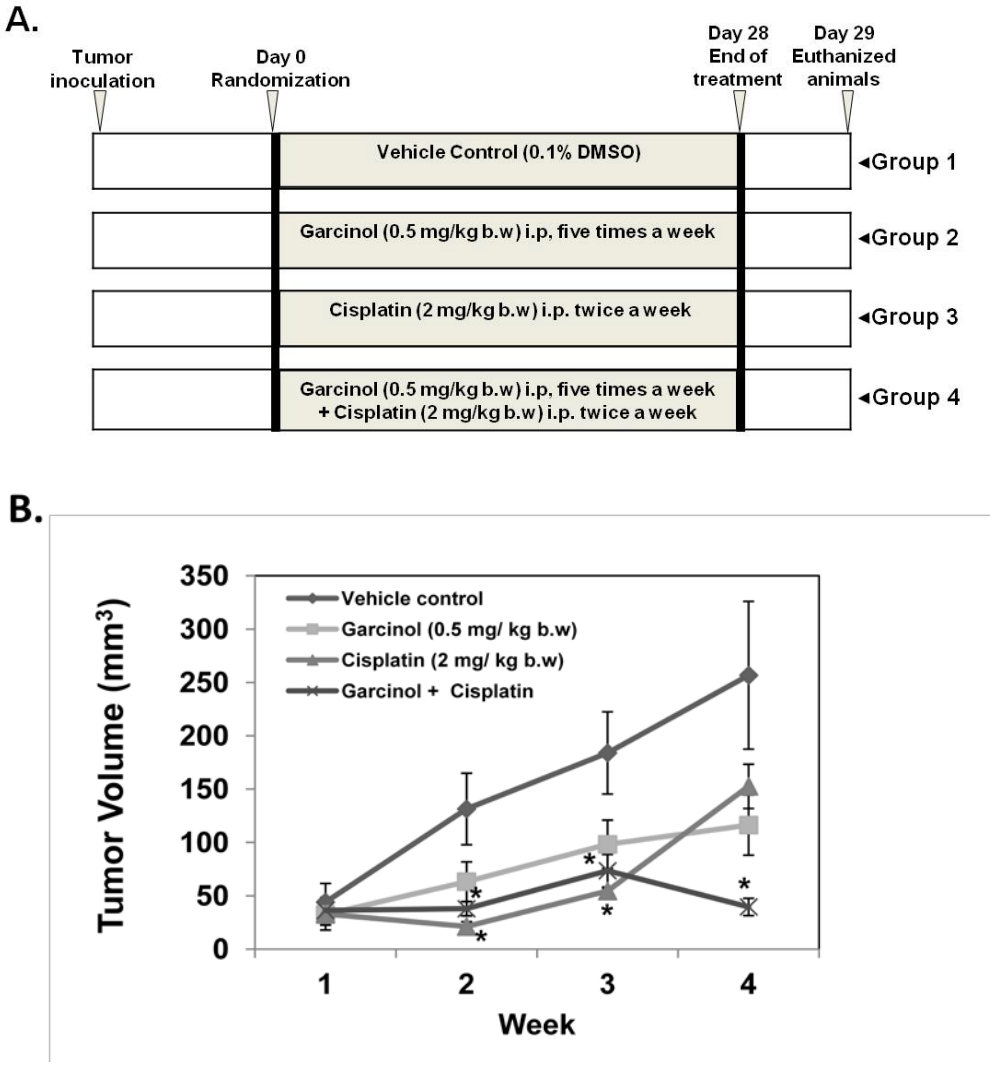


Figure 3.6.3.1. Garcinol enhances the inhibition of tumor growth induced by cisplatin *in vivo*.

A. Schematic representation of experimental protocol described in "Materials and Methods". Group I was given 0.1% DMSO, group II was given garcinol (0.5 mg/kg body weight), group III was given cisplatin (2 mg/kg body weight), and group IV was given garcinol (0.5 mg/kg) and cisplatin (2 mg/kg).

B. The diameters of the tumor were measured twice per week, after which the tumor volume was calculated as: (longest diameter) × (shortest diameter)^2) × 0.5. (* $p < 0.05$)

3.6.3.2 Garcinol enhances the effects of cisplatin in abrogating Ki-67 and CD31 expression in HNSCC tumor tissues.

As evident from our previous observations garcinol treatment alone suppressed the expression of proliferation marker Ki-67 and microvessel density marker CD31 *in vivo*, we next determined whether garcinol in combination with cisplatin could also exert similar effects. Tumor samples from garcinol and or cisplatin treated xenograft mice were evaluated by immunohistochemistry. We found that both garcinol and cisplatin downregulated the expression of Ki-67 in CAL27 tissue to a similar extent, and the two agents when used in combination together were most effective ($p < 0.01$ versus vehicle; $p < 0.01$ versus cisplatin alone). Similarly, both agents significantly inhibited CD31 expression alone, and the maximum decrease was noted when the two drugs were used in combination ($p < 0.01$ versus vehicle; $p < 0.01$ versus cisplatin alone).

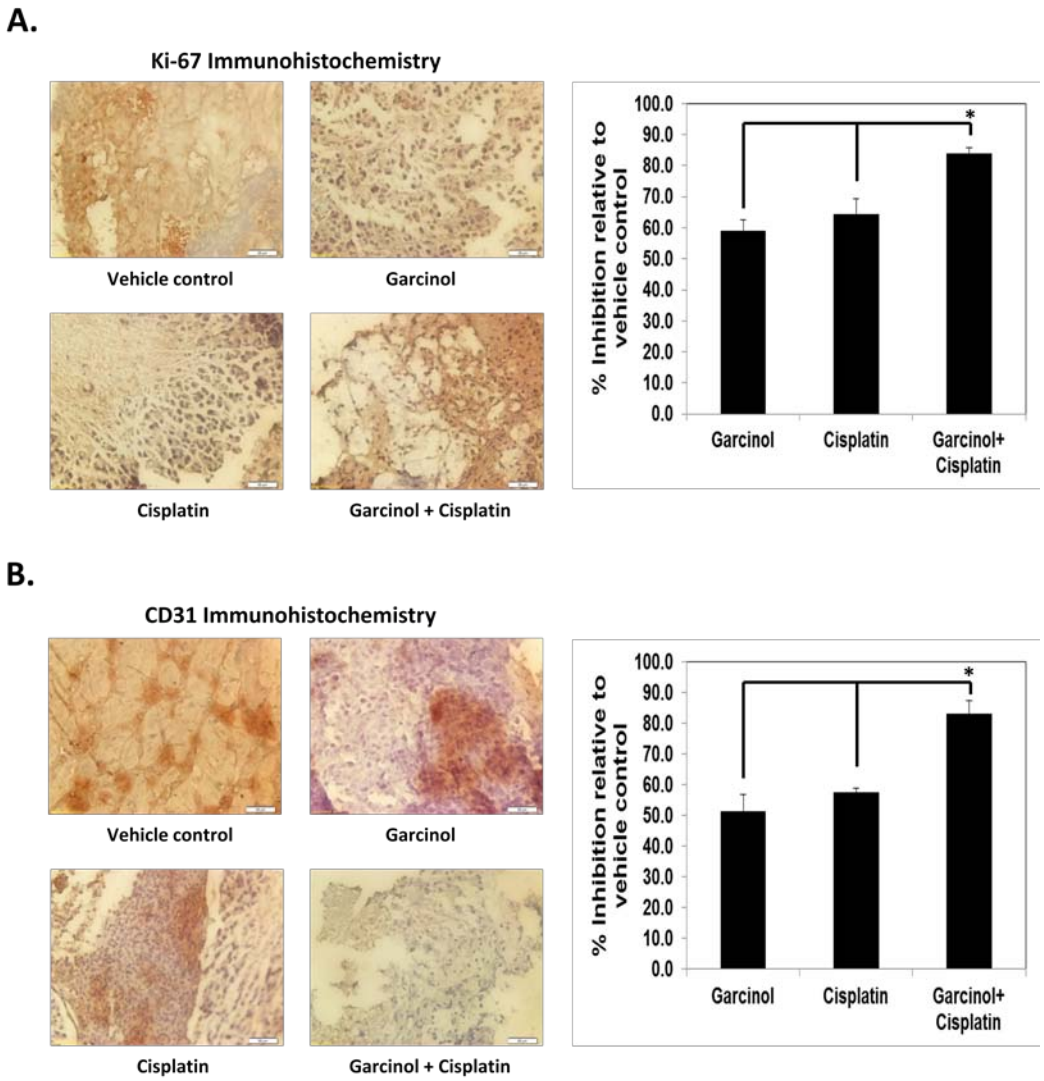


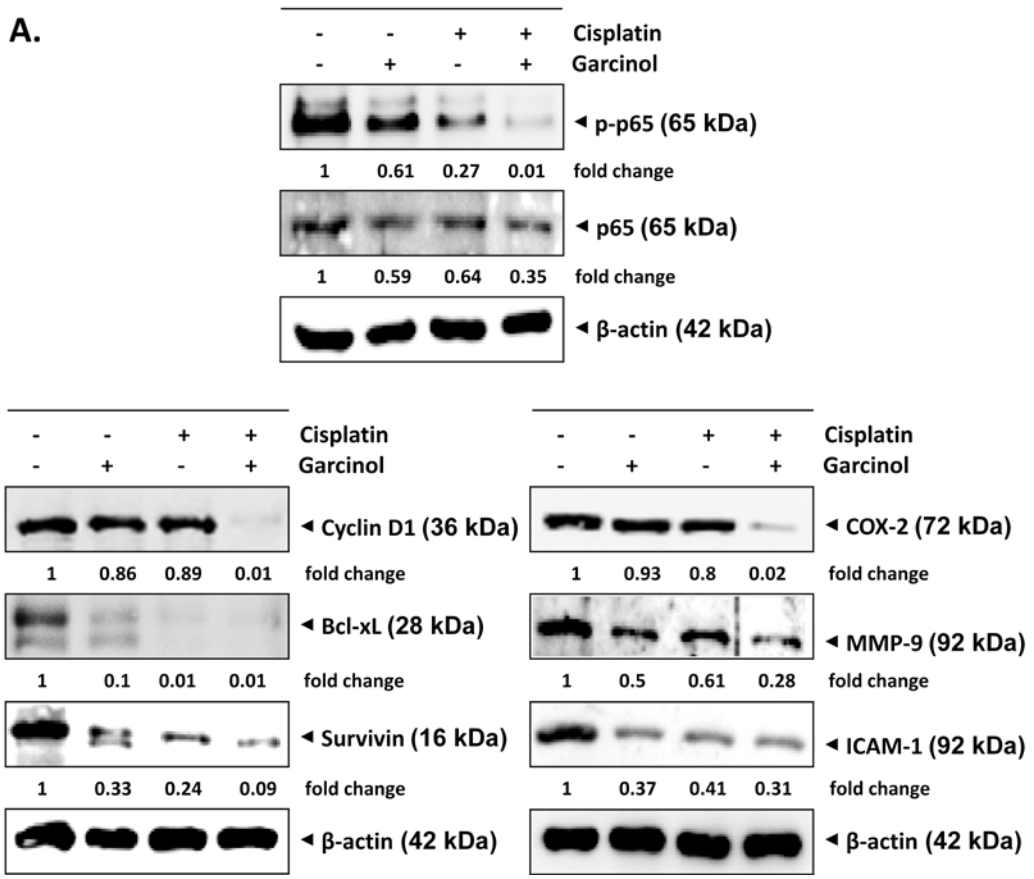
Figure 3.6.3.2. Garcinol enhances the effect of cisplatin against tumor cell proliferative and angiogenesis biomarkers in HNSCC tumor tissues.

A. *Left panel*, Tumor tissues obtained were subjected to immunohistochemistry using Ki-67 antibody. Representative image for each group is shown above. *Right panel*, quantification of Ki-67+ cells.

B. *Left panel*, Tumor tissues obtained were subjected to immunohistochemistry using CD31 antibody. Representative image for each group is shown above. *Right panel*, quantification of CD31+ microvessel density.

3.6.3.3 Garcinol in combination with cisplatin downregulates the expression various oncogenic molecules involved in HNSCC progression.

From our *in vitro* data, we found that garcinol suppressed both the constitutive and inducible NF-κB activation in HNSCC cell lines. Hence, we next evaluated the effects of garcinol and cisplatin on NF-κB levels and expression of various NF-κB-regulated gene products in HNSCC tumor tissues. Tumor tissue samples harvested from drug treated xenograft mice were analyzed by western blot and immunohistochemistry analysis. Western blot analysis of nuclear extract of tumor tissue showed that garcinol either alone or in combination with cisplatin was effective to suppress the constitutive NF-κB (p65) expression in HNSCC tumor tissues. The results of western blot analysis also clearly showed that the combination treatment of garcinol and cisplatin also substantially downregulated the expression of COX-2, VEGF, and MMP-9 proteins. The IHC analysis results further supported the data obtained from western blotting assay.


A.
- - + + Cisplatin
- + - + Garcinol
◄ p-p65 (65 kDa)
1 0.61 0.27 0.01 fold change
◄ p65 (65 kDa)
1 0.59 0.64 0.35 fold change
◄ β-actin (42 kDa)
- - + + Cisplatin
- + - + Garcinol
◄ Cyclin D1 (36 kDa)
1 0.86 0.89 0.01 fold change
◄ Bcl-xL (28 kDa)
1 0.1 0.01 0.01 fold change
◄ Survivin (16 kDa)
1 0.33 0.24 0.09 fold change
◄ β-actin (42 kDa)
- - + + Cisplatin
- + - + Garcinol
◄ COX-2 (72 kDa)
1 0.93 0.8 0.02 fold change
◄ MMP-9 (92 kDa)
1 0.5 0.61 0.28 fold change
◄ ICAM-1 (92 kDa)
1 0.37 0.41 0.31 fold change
◄ β-actin (42 kDa)

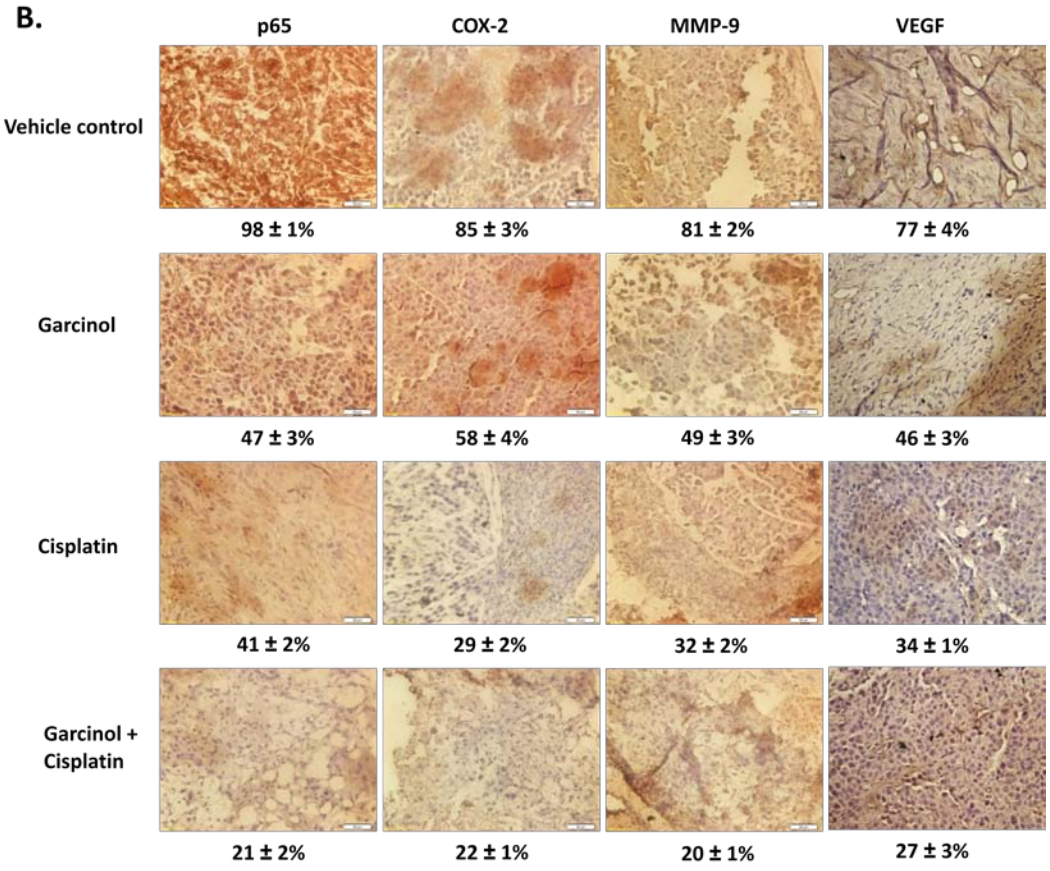


Figure 3.6.3.3. Garcinol in combination with cisplatin suppresses NF-κB activation and the expression of NF-κB-regulated gene products in HNSCC tumor tissues.

A. Proteins extracted from the all four treatment groups were subjected to western blot using various antibodies as indicated. β-actin as an internal control to verify equal loading of proteins was detected on the same membrane after stripping. Samples from three mice in each group were analyzed and representative data are shown.

B. Tumor tissues obtained were subjected to immunohistochemistry using antibodies against NF-κB, COX-2, VEGF, and MMP-9 proteins. Representative image for each group is shown above.

4 DISCUSSION

4.1 General discussion

Despite the recent medical advances in the development of sophisticated systemic therapy, the five-year survival rate of advanced HNSCC remains low with minimal improvement over the past decades (132). Multidisciplinary approaches involving surgical excision, radiation therapy and chemotherapy are applied for the treatment of HNSCC, but frequently failed due to chemoresistance, tumor recurrences and distant metastases in locoregionally advanced disease (8). Platinum compounds such as cisplatin and carboplatin are used as the first-line chemotherapy for the treatment of HNSCC, although many other agents have also been extensively studied (138, 139). A meta-analysis of a panel of clinical trials has revealed that platinum containing regimens exhibit higher response rates than non-platinum therapies (266). Unfortunately, only 20 to 40% of the patients experience complete response to platinating agents, mainly because of the intrinsic or acquired resistance to chemotherapy (150). High dose of platinating agents which are required for significant antitumor effect lead to frequent and severe side effects such as nausea, vomiting, mucositis, neurotoxicity, and renal dysfunction that compromise quality of life of the patients (148, 267) (Table 3).

For the above reasons, novel agents with lower toxicity which can enhance the effects of current chemotherapeutic drugs and overcome the chemoresistance are in great need.

Natural products for ancient medical uses such as t raditional Chinese medicine and Ayurveda practiced in India are gaining increasing patronage for the treatment of cancer in recent years (169, 268). Garcinol, a poly-isoprenylated benzophenol, is one such compound extracted from the dried rind of the fruit of *Garcinia indica* tree which is used as a traditional folk medicine for the treatment of inflammatory conditions as diverse as rheumatism, edema, ulcer and infectious diseases (173). Garcinol has gained much attention in the field of cancer research in recent years, and several reports have demonstrated its significant anti-neoplastic and chemopreventive roles of garcinol in different *in vitro* and *in vivo* models.

4.1.1 Garcinol inhibits cell viability and induces programmed cell death in HNSCC cells.

It is well documented that most anticancer agents induce cell cycle arrest and/ or apoptosis (229, 269). As described earlier, garcinol has been reported to inhibit cell proliferation and induce apoptosis in a various types of tumor cells but not in HNSCC cell lines previously. Using MTT assay, we confirmed the ability of garcinol to significantly suppress the viability of human HNSCC cell lines in a time- and dose- dependent manner. Interestingly, garcinol exhibited minimal cytotoxic effects against non-cancerous cells normal liver (LO2) and breast epithelial (MCF10A), cell lines, thereby indicating its specificity towards tumor cells (data not shown). Prior studies have also reported that garcinol exerts potent growth inhibitory effects on c olon cancer (HT-29 and HCT-116) and breast cancer cell lines (MDA-MB-231 and MCF-7), but much less effective in inhibiting the growth of normal immortalized intestinal cells (IEC-6 and INT-407) and normal breast epithelial cell line (MCF-10A) (198, 270).

The inhibition of HNSCC cell viability by garcinol could be attributed to the ability of garcinol to suppress the cell proliferation, induce apoptosis, or a combination of the two. The most accurate method to measure cell proliferation is

by directly measuring DNA synthesis. Conventionally, the detection was achieved by incorporation of radioactive deoxynucleoside such as [^{3}H] thymidine or thymidine analogue bromo-deoxyuridine (BrdU) (271, 272). The use of EdU as a novel alternative to the BrdU assay allows faster, more efficient detection by a fluorescent azide through direct conjugation to the thymidine analogue (271). Our data confirmed the ability of garcinol to inhibit the HNSCC cell proliferation in a time- and dose-dependent manner.

To investigate whether the garcinol-induced cell death was due to the apoptosis, multiple approaches have been employed. We observed that garcinol caused substantial increased accumulation of HNSCC cells in sub-G1 population, induced DNA fragmentation and increased the percentage of annexin V-positive cells, providing solid evidences that cell death induced by garcinol is apoptotic in nature. The apoptosis induced by garcinol was caspase-3 dependent as evidenced by the elevated level of active (cleaved) caspase-3. Moreover, we noticed that the garcinol-induced apoptosis involved both caspase-8 mediated extrinsic and caspase-9 mediated intrinsic apoptotic pathways. Our findings are in accordance with the observations that garcinol-induced substantial activation of caspase-8 and caspase-9 in diverse pancreatic, colon and liver cancer cell lines (199, 231, 233).

4.1.2 Garcinol modulates the expression of cell cycle regulator proteins.

D type cyclins form complex with function as regulatory subunits of CDK that control the transition from G1 to S phase of the cell cycle (273, 274). Dysregulation of cyclin D1 alters cell cycle progression, results in uncontrolled cell proliferation as the hallmark of cancer (275). Abnormal expression of cyclin D1 has been implicated in the pathogenesis of several types of cancers including HNSCC (276). It is evident that garcinol suppressed the expression of cyclin D1 in HNSCC cells in a time-dependent manner. Cyclin E is a late G1 cyclin, which, along with its catalytic subunit CDK2, phosphorylates the CDK inhibitor $p27^{Kip1}$ and bring it to destruction in proteasomes (273). The high levels of cyclin E and low levels of p27 are indicative of reduced long-term survival and increased mortality in various forms of cancer (273, 277). Similarly, we also found that garcinol downregulated the expression of cyclin E, and increased the level of p27 protein in a time-dependent manner. The inhibition of cyclin D1 and cyclin E expression, together with the upregulation of p27 may account for garcinol's ability to significantly inhibit the proliferation of various HNSCC cell lines.

4.1.3 Garcinol alters the balance between pro-apoptotic and anti-apoptotic proteins to induce apoptosis.

The balance between pro-apoptotic and anti-apoptotic proteins of Bcl-2 family determines the cell fate whether they undergo apoptosis (278, 279). We found that garcinol downregulated the protein and mRNA expression of various anti-apoptotic molecules, namely Bcl-2, Bcl-xL and Mcl-1, in concordance with several prior studies (217, 231-233). Most of the above studies have also observed that garcinol could increase the expression of anti-apoptotic protein Bax. On the contrary, our results revealed that the expression levels of Bax and Bak remained largely unaltered upon garcinol treatment, in accordance with the experimental findings by Liao and coworkers (217). Inhibitor of apoptosis protein (IAP) family is another group of important negative regulators of apoptosis (280). For example, survivin and XIAP can bind the effector caspase-3, -7 and -9 and inhibit the caspase activity (280, 281), whereas both of the two isoforms of c-FLIP block caspase-8 activation (282). Interestingly, we found that garcinol downregulated these above mentioned direct inhibitors of caspases; and together with the garcinol-induced increase of Bax (Bak)/Bcl-2 (Bcl-xL) ratio, resulted in enhanced apoptosis.

4.1.4 Garcinol suppresses the expression of proteins involved in angiogenesis.

Tumor-associated neovasculature, generated by the process of angiogenesis develops during tumorogenesis to facilitate tumor acquiring nutrients and oxygen and disposing metabolic wastes (4). This process is initiated by 'angiogenic switch' in which the balance between pro-angiogenic and anti-angiogenic factors is tipped towards angiogenesis (283). HNSCC cells have been found to express and secret pro-angiogenic factors such as the major contributor to angiogenesis VEGF, and various growth factors (e.g. PDGF) and cytokines (e.g. IL-8), which are usually associated with poor prognosis (253, 284). We examined the effects of garcinol on VEGF in HNSCC cell lines and observed a decreased expression of VEGF at both the protein and mRNA level. Interestingly, the decrease in this angiogenic growth factor, in turn, correlated with a significant decrease in microvessel density biomarker CD31 as observed in our *in vivo* study. Taken together, these findings suggest a potential ability of garcinol to inhibit angiogenesis, thereby, abrogating HNSCC progression.

4.1.5 Garcinol exerts anti-migratory and anti-invasive effects against HNSCC cells.

Tumor cells are known to secrete MMPs, which are thought to degrade extracellular matrix (ECM) and facilitate tumor cell invasion in tissues, that MMP-9 and MMP-2 is well established to play the most important role in the process of epithelial-mesenchymal transition (EMT) (285). The cell adhesion molecule, ICAM-1 has been implicated in various stages of tumor progression and metastasis (286, 287). Functional CXC chemokine receptor 4 (CXCR4) is usually preferentially expressed in high level in certain metastatic HNSCC tissues and promotes the establishment of lymph node metastasis (288-290). In xenograft mouse model, CXCR4 antagonist blocks both growth of primary tumor and metastasis of HNSCC (291). Moreover, the sole ligand of CXCR4, chemokine CXCL12, has been found to induce CXCR4 mediated MMP-9 secretion in HNSCC (292). Interestingly, our experimental findings showed that CXCL12 induced a significant migration and invasion of HNSCC cells. We observed that garcinol effectively attenuated the process of CXCL12-induced migration and invasion, that are in agreement with the observations found in colon, breast cancer, prostate and pancreatic cancer cell lines (199, 201, 204, 217). The significant

reduction of tumor cell migration and invasion directly correlated with the garcinol-induced downregulation of MMP-9, MMP-2, ICAM-1 and CXCR4 proteins. Overall, our data clearly established the anti-invasive potential of garcinol through the suppression of above indicated oncogenic molecules in HNSCC cells.

4.2 Garcinol inhibits the STAT3 signaling cascade in HNSCC cells.

The activation STAT3 signaling in cancer cells regulates the expression of numerous genes to supports tumor cell proliferation, survival, angiogenesis and metastasis (70, 293). As garcinol treatment in HNSCC cells resulted in suppression of cell growth, induction of apoptosis, along with the downregulation of various gene products ; we also investigate whether garcinol exhibit its anti-neoplastic effects by modulating STAT3 signaling pathway in HNSCC cells. Our findings showed that garcinol indeed suppressed both constitutive and IL-6 induced STAT3 phosphorylation at tyrosine 705 residue in a variety of HNSCC cells. In addition to canonical tyrosine phosphorylation, STAT3 is also phosphorylated at serine residue regulating the transcriptional activity. We conducted western blot using serine phosphorylation specific antibody to explore the status of serine phosphorylation in HNSCC cells upon garcinol treatment. We observed that garcinol did not alter the level of serine phosphorylation, indicating that garcinol specifically inhibited tyrosine phosphorylation of STAT3. Next, we noticed that the garcinol-induced inhibition of STAT3 occurs in parallel with the suppression of the upstream kinases (Src, JAK1, and JAK2). Prior reports have revealed that Src and JAK2 act cooperatively to regulate the constitutive

activation of STAT3 (261, 294). Our data suggested that garcinol may block the cooperation of Src and JAK kinases regulating STAT3 phosphorylation. Tyrosine phosphatases negatively regulate STAT3 activation by dephosphorylating the signaling molecules in the STAT3 pathway (73, 295). Many physiological inhibitors of STAT3 and natural compounds have been reported to suppress JAK/STAT signaling by upregulating the expression of PTPs, or promoting the phosphatase activities (296-298). Thus, we also explored the possible involvement of tyrosine phosphatases in garcinol-induced inhibition of STAT3 activation. However, treatment with garcinol did not affect the expression and/or phosphorylation of the important PTPs in HNSCC cells, suggesting that this benzophenone inhibitory effect on STAT3 signaling cascade is not mediated via induction of phosphatases. Also, whether garcinol affects other STAT3 inhibitors such as protein inhibitors of activated STAT3 (PIAS3) and suppressor of cytokine signaling (SOCS) in HNSCC cells needs further investigation. STAT3 monomers form dimers upon tyrosine phosphorylation, and then translocate to the nucleus where they bind to specific DNA to activate transcription (70). We noted that garcinol negatively regulated STAT3 signaling cascade at multiple steps as experiments as STAT3 nuclear translocation, DNA-binding ability and IL-6/EGF

induced reporter activity of STAT3 were significantly abrogated upon garcinol treatment in HNSCC cells.

4.3 Garcinol suppresses NF-κB signaling pathway in HNSCC cells.

It has been reported previously that aberrant NF-κB signaling contributes to different stages in HNSCC initiation and progression (123-125). And our experimental findings have demonstrated that garcinol inhibited cell viability, induced apoptosis, and abrogated processes of migration and invasion, in parallel with downregulating the expression of various oncogenic proteins involved in cell cycle, survival, invasion, and angiogenesis. We hypothesized that inhibition of NF-κB signaling cascade by may at least in part contribute to its observed anti-neoplastic effects in HNSCC cells. We first found that NF-κB is constitutively active in HNSCC cells, but the exact reason, why it is persistently active is still not clear. Our results showed that garcinol suppressed phosphorylation and degradation of the constitutive IκBα, thereby prevented the nuclear translocation of NF-κB p65 subunit as evidenced by decreased p65 level in the nucleus. Optimal NF-κB activation requires phosphorylation on functional domains of NF-κB proteins, for example, phosphorylation of p65 a t Ser536 enhance p65 transactivation potential in most cases (299). We also noticed that garcinol suppressed p65 phosphorylation at Ser 536 residue in a time-dependent manner in HNSCC cells. Consistently, by E LISA-based Trans AM™ NF-κB assay, we

found that treatment of HNSCC cells with garcinol inhibited DNA binding ability of NF-κB. The inhibition of IKK activation by garcinol suggests that it abolished NF-κB activation in HNSCC cells indirectly through the suppression of IKK phosphorylation. Several kinases, such as Akt, mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), MEKK3, protein kinase C (PKC), GSK-3β, phosphoinositide-dependent protein kinase-1 (PDK1), and TAK1, (300), have been reported to function upstream of IKK. We also reported for the first time that garcinol could inhibit the constitutive TAK1 activation in HNSCC cells. Moreover, a prior study indicates that the constitutive activation of NF-κB in HNSCC cells is mediated through the TRADD-TRAF2-RIP-TAK1-IKK pathway (301), and our results clearly indicate that garcinol can indeed abrogate TAK1-mediated IKK activation.

Previous studies have also indicated that several chemotherapeutic agents including platinum compounds induce the activation of NF-κB signaling in protection from genotoxic stress, which lead to the drug resistance in tumor cells (115, 264, 302). For example, cisplatin has been shown to significantly induce NF-κB promoter activity, accompanied with the increase in nuclear p65 and p50 protein levels in HNSCC cell lines (303). However, the role of NF-κB in

chemoresistance remains controversial (304). Interestingly, we found that cisplatin can substantially increase nuclear p65 expression and phosphorylation level as well as NF-κB DNA binding activity in HNSCC cells, and such cisplatin-induced NF-κB activation could also be effectively suppressed by garcinol. A recent report has suggested that NF-κB can also mediate cisplatin-induced resistance through histone modifications in HNSCC cells (265), and thus inducible NF-κB inhibitory effects of garcinol may be mediated through its previously documented potent histone acetyltransferases blocking effects as observed in other tumor cells (188). Our results are also in part agreement with another study in which curcumin was also found to enhance the effect of cisplatin in suppression of HNSCC via inhibition of IKKβ protein of the NF-κB pathway (305). We further observed that cisplatin also substantially induced the expression of the oncogenic MMP-9, ICAM-1 and COX-2 proteins, and garcinol treatment was able to suppress both constitutive and cisplatin-induced expression of these diverse NF-κB-regulated gene products involved in HNSCC progression.

Different modes of functional interaction between STAT3 and NF-κB have been discovered in tumors. One interesting study had shown that STAT3 could prolong NF-κB nuclear retention through acetyltransferase p300-mediated RelA

acetylation, thereby interfering with NF-κB nuclear export (306). Thus, it is possible that suppression of STAT3 activation may mediate the inhibition of NF-κB activation by garcinol. Also, the constitutive activation of NF-κB in HNSCC leads to the production and secretion of cytokines such as IL-6 in an autocrine/paracrine manner, which causes the consequent activation of STAT3 signaling (307). Importantly, STAT3 and NF-κB control both distinct and overlapping groups of genes involved in cell proliferation, survival, angiogenesis and invasion (308, 309). Our work has demonstrated that garcinol inhibited both constitutive and inducible STAT3/NF-κB activation and downregulated various STAT3/NF-κB-regulated genes, as one of the mechanisms behind the observations of garcinol-induced apoptosis, inhibition of cell viability, and the abrogation of tumor cell invasion and migration in HNSCC cells. Although previous studies have shown that garcinol can modulate AKT survival signaling cascade in colorectal cancer cells (217), our study is also the first report to examine the effect of garcinol on Akt/mTOR/S6K1 signaling axis in HNSCC cells. We found that garcinol inhibited the phosphorylation of Akt, mTOR, and S6K1 in a time-dependent manner in HNSCC cells, which further indicates the possibility that garcinol could interfere with the mTOR-rictor complex that

phosphorylates Akt at Ser473. Akt is also reported to regulate the master transcription factor NF-kB through the phosphorylation of p65 to enhance the transcriptional activity of NF-kB (310). Furthermore, Akt is also involved in the regulation of IL-6 induced STAT3 signaling pathway (261). Thus, it is possible that the inhibition of AKT/mTOR/S6K pathways may contribute to STAT3/NF-κB inhibitory effects of garcinol as observed in HNSCC cells.

4.4 Garcinol potentiates the apoptosis induced by cisplatin in HNSCC cells.

There are only few published reports in which garcinol have been used in combination with existing neoplastic agents. Garcinol has been shown to potentiate the apoptotic effects of natural ligand TRAIL in colon cancer (231), and natural compound curcumin in pancreatic cancer (237). And only one study by Parasramka's group has demonstrated that garcinol significantly sensitized pancreatic cancer cells to the first-line pancreatic cancer drug, gemcitabine (221). However, there are no prior reports investigating the potential of garcinol as chemosensitizing agent against HNSCC cell lines. Our study showed for the first time that garcinol when used at sub-optimal concentrations significantly enhanced the cytotoxic and apoptotic effects of the chemotherapeutic drug cisplatin against a number of HNSCC cell lines. Also, cisplatin was been found to significantly induce NF-κB activation as evidenced by the increased nuclear p65 protein and phosphorylation level, and enhanced NF-κB DNA binding activity; and both the constitutive and cisplatin-induced NF-κB activation could be suppressed by garcinol. Therefore, it is conceivable that garcinol attenuated NF-κB-mediated drug resistance that may lead to the observed enhanced apoptotic effect. Also, the pleiotropic nature of garcinol with its ability to modulate multiple signaling

pathways including STAT3 and Akt cascades as reported above, may contribute to its potent anticancer effects.

4.5 Anti-tumor effects of garcinol in HNSCC xenograft mouse model

Whether the *in vitro* observations with garcinol have any relevance to the *in vivo* context was also investigated.

4.5.1 Pharmacokinetic properties of garcinol

In order to examine the efficacy of garcinol in a xenograft mouse model, a pharmacokinetic study of garcinol was conducted to define suitable doses for treatment of mice in pharmacodynamics study. Two doses of garcinol (0.5 and 2 mg/kg) were used for evaluation of pharmacokinetic property of garcinol through intraperitoneal administration. The pharmacokinetic results indicated that garcinol showed a sufficient systemic exposure and good dose proportionality which is an important property for garcinol in its translational development from animal to human subjects. The pharmacokinetic results suggested that 0.5 and 2 mg/kg of garcinol are the suitable doses for its pharmacodynamics evaluation.

4.5.2 Garcinol inhibits the growth of HNSCC xenograft tumor.

Our results indeed indicate for the first time that garcinol significantly inhibited HNSCC growth in a nude mouse model without exhibiting any significant toxicity, and downregulated the expression of proliferation marker Ki-67, and microvessel density index CD31. To the best of our knowledge, no prior studies with garcinol in xenograft HNSCC models have been reported previously, and thus our novel findings suggest that garcinol may have a tremendous potential for the treatment of HNSCC. Interestingly, garcinol has been tested before in chemically-induced HNSCC models, and presented chemopreventive effects against 4-NQO-induced tongue cancer in rats (245) and DMBA-induced oral cancer in hamster cheek pouch (211); however, its activity against HNSCC xenograft model remains unexplored till date.

4.5.3 Garcinol potentiates the inhibition of tumor growth induced by cisplatin in nude mice.

Up to date, there is only one *in vivo* combination study published recently, revealing that garcinol enhances the effect of targeted therapy (anti-ERBB2 antibody) against breast cancer in a syngeneic tumor model (190). However, the effects of garcinol in combination with anticancer therapies in HNSCC mouse models have never been evaluated before. When examined in xenograft mouse model, garcinol or cisplatin alone was found to inhibit the tumor growth effectively, and the combination of these two agents markedly augmented the effect to a greater extent. This observation was in parallel with the enhanced suppression of Ki-67 and CD31 observed in the tumor tissues with the drug combination, thereby indicating that garcinol enhanced the inhibition of proliferation and angiogenesis in combination with cisplatin. This is the first study to systematically analyze the effects of garcinol in combination with an approved chemotherapy (cisplatin) *in vivo* in a tumor model of HNSCC.

4.5.4 Garcinol inhibits the expression of STAT3 and NF-κB in HNSCC tumor tissues.

We also investigated whether the effects of garcinol induced tumor growth retardation in mice were mediated by the abrogation of both STAT3 and NF-κB activation cascades. As shown by I HC analysis, garcinol downregulated the expression of activated STAT3 and NF-κB expression in the garcinol treated groups as compared with the control group. These experimental findings are in agreement with the studies showing that the anti-tumor action of garcinol in xenograft breast cancer and hepatocellular carcinoma models is mediated through inhibition of STAT3 signaling (204, 311). However, the effects of garcinol on NF-κB activation cascade are not well characterized in *vivo* models. Our studies demonstrate for the first time that garcinol in combination with cisplatin further suppressed p65 expression, which was associated with greater inhibition of tumor growth.

4.5.5 Garcinol in combination with cisplatin downregulates the expression of proteins involved in HNSCC progression *in vivo.*

In order to further validate the suppressive effects of garcinol on NF-κB signaling observed in HNSCC cell lines, we performed western blot and IHC analysis to examine whether garcinol-cisplatin combination modulates NF-κB-regulated gene products involved in HNSCC progression in a xenograft mouse model. The analysis on human HNSCC tumor tissues demonstrated that garcinol in combination with cisplatin significantly suppressed the overexpression of critical oncogenic molecules involved in proliferation (cyclin D1, COX-2), survival (Bcl-xL, survivin), angiogenesis (VEGF) and invasion (MMP-9, ICAM-1), and such downregulation was more impressive than observed with either garcinol or cisplatin alone. Overall, our findings clearly demonstrate that garcinol significantly suppresses the activation of NF-κB signaling cascade thereby inhibiting the expression of various oncogenic biomarkers of proliferation, survival, angiogenesis, invasion, and metastasis.

5 CONCLUSIONS

In summary, our studies demonstrated that the garcinol, an active component of *Garcinia indica*, suppressed HNSCC cell viability, induced programmed cell death and inhibited the processes of cellular migration and invasion; the observed anti-cancer effects of garcinol are correlated with the donwregulation of various gene products involved in diverse aspects of HNSCC growth, survival, angiogenesis, invasion, and metastasis. We then identified STAT3 and NF-κB as the important signaling cascades being modulated upon garcinol treatment in HNSCC cells. Garcinol suppressed both constitutive and IL-6-induced STAT3 activation in parallel with the suppression of the upstream kinases (c-Src, JAK1, and JAK2) in HNSCC cells, while the polyisoprenylated benzophenone inhibited NF-κB signaling through the modulation of TAK1-mediated IKK activation. The modulatory effect of garcinol on these pathways was further verified by t he downregulation of p-STAT3 and p65 expression in tumor samples from garcinol treated xenograft mice. Thereafter, we carried out experiments to evaluate the potential of garcinol in sensitizing the HNSCC to cisplatin treatment both in HNSCC cell lines and in xenograft mouse model. Interestingly, we observed that cisplatin induced NF-κB activation and upregulated the expression of various NF-

κB-regulated gene products, implying a plausible mechanism for the frequently occurred resistance to this HNSCC first-line chemotherapeutic agent. Also, garcinol was able to suppress the cisplatin-induced NF-κB activation, downregulate the expression of various NF-κB-regulated proteins, and potentiate the cisplatin-induced apoptosis in HNSCC cells. Furthermore, our i*n vivo* study showed that the combination treatment of garcinol and cisplatin significantly reduced the growth of HNSCC xenograft tumors in nude mice. Further analysis of tumor tissues demonstrated that garcinol inhibited NF-κB, as well as the target gene products involved in proliferation (cyclin D1, COX-2), survival (Bcl-xL, survivin), angiogenesis (VEGF) and invasion (MMP-9, ICAM-1); moreover, it exerted significant enhanced effects when combined with cisplatin. Overall, our results clearly validate the anti-cancer effects of garcinol in HNSCC are mediated through suppression of multiple pro-inflammatory signaling cascades; the experimental evidences of garcinol's capability to potentiate the effects of cisplatin provide a solid rationale for pursuing the application of garcinol further to enhance treatment efficacy for HNSCC.

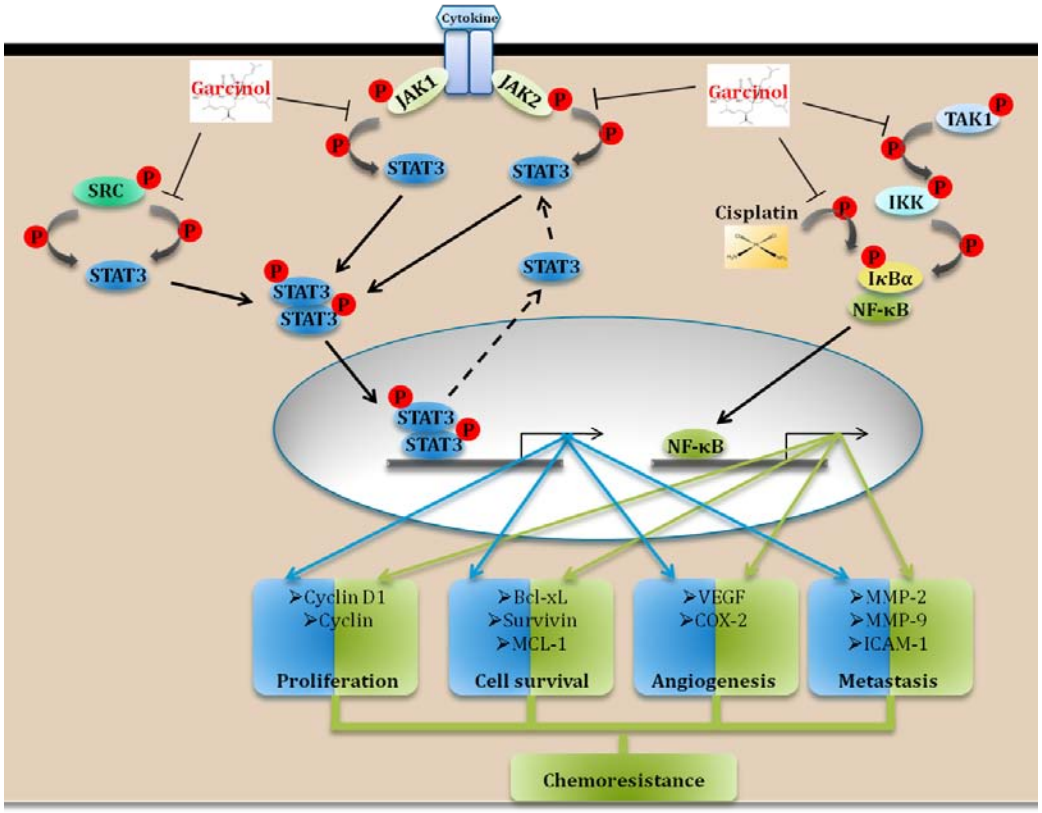


Figure 5.1. Schematic diagram representing the molecular mechanism(s) of garcinol against HNSCC.

Table 1: Selected ongoing clinical trials in HNSCC patients

Clinical status	Drug	Mechnism of Action	Identifier No.
Chemoprevention			
Phase II	**Celecoxib** + Erlotinib	COX-2 inhibitor	NCT00400374
Phase II	**Soy Isoflavones**	Natrual product	NCT02007200
Phase III	**Erlotinib (Tarceva)** vs Placebo	TKI (EGFR)	NCT00402779
Phase III	**α-tocopherol and β-carotene supplements** vs placebo	Natrual product	NCT00169845
Treatment			
Phase I/II	**PX-866** + Cetuximab vs Cetuximab	PI3K inhibitor	NCT01252628
Phase I/II	**MLN8237 (Alisertib)**	Aurora A kinase inhibitor	NCT01045421
Phase II	**MK-2206**	Akt inhibitor	NCT01370070
Phase II	**Dalantercept**	ALK1 inhibitor	NCT01458392
Phase II	**Rapamycin (*Sirolimus*)**	mTOR inhibitor	NCT01195922
Phase II	**Vorinostat** + Capecitabine vs Capecitabine	Histone deacetylases (HDAC) inhibitor	NCT01267240
Phase I/II	**E7050 (golvantinib)** + Cetuximab vs Cetuximab	TKI (c-Met and VEGFR-2)	NCT01332266
Phase II	**Dasatinib (BMS-354825)**	TKI (BCR/Abl and Src)	NCT00859937
Phase II	**Axitinib (Inlyta)**	TKI (VEGF, c-KIT and PDGFR)	NCT01469546
Phase III	**Afatinib** vs Methotrexate	TKI (EGFR and HER2)	NCT01345682
Phase II	**Bevacizumab (Avastin)** + Cisplatin	mAb (VEGF)	NCT00423930
Phase III	**Cetuximab (Erbitux)** + Cisplatin vs Cisplatin	mAb (EGFR)	NCT00265941
Phase III	**Fluorouracil** + Cisplatin vs Cisplatin	Thymidylate synthase (TS) inhibitor	NCT00608205
Phase III	**Docetaxel** + Cisplatin + Fluorouracil vs Cisplatin + Fluorouracil	Microtubule inhibitor	NCT00995293

Abbreviations: **TKI**, tyrosine kinase inhibitor; **mAb**, monoclonal antibody

Table 2: Selected successful clinical trials with natural products in cancer patients

Name	Source	Clinical status	Disease	Effects	Ref	Remarks
Curcumin	*Curcuma longa*	Phase IIa	Colorectal neoplasia	Reduced aberrant crypt foci (ACF) formation in smokers	(319)	
		Phase II	Advanced pancreatic cancer	well tolerated, presented biological activity in some patients	(320)	
Genistein	*Genista tinctoria*	Phase II	Prostate cancer	Decrease in serum prostate specific antigen (PSA) levels	(321)	
			Prostate cancer	Reduced incidence of prostate cancer. Arm A: 28.0%; Arm B: 57.1%)	(322)	Arm A: placebo group Arm B: isoflavone group
Flavopiridol (alvocidib)	*Dysoxylum binectariferum*	Phase II	Relapsed chronic lymphocytic leukemia (CLL)	PRs 47%; nodular PRs 5%; CRs 1.6% Majority of genetically high-risk patients responded	(323)	
			Poor-risk acute myelogenous leukemia (AML)	Yielded 67% complete remission. Arm A: 62%; Arm B: 74%	(324)	Arm A: bolus flavopiridol Arm B: flavopiridol folowed by ara-C and mitoxantrone
Polyphenon E extract	Green tea	Phase II	Patients with high risk oral premalignant lesions (OPL)	Higher OPL clinical response rate. Arm A: 50%; Arm B: 18.2%	(325)	Arm A: green tea extract group Arm B: placebo group
			Chronic lymphocytic leukemia (CLL)	Declines in the absolute lymphocyte count and/or lymphadenopathy	(326)	

Table 3: Toxicities associated with cisplatin-based chemotharepy in HNSCC patients

Disease	Clinical status	Arm A	Arm B	Arm C	Major toxicities (Grade≥3)	Percentage	Ref
Advanced HNSCC	Phase II	CRT (P)			Hematologic toxicity	34%	(312)
					Dysphagia	62%	
					Mucositis/stomatitis	53%	
					Nausea and vomiting	37%	
Locoregionally advanced HNSCC	Phase II	CRT (PF)			Mucositis	57%	(313)
					Neutropenia	39%	
					Thrombocytopenia	53%	
Advanced HNSCC	Phase III	CT (PF)	CT (PT)		Leukopenia	A 63%; B 35%	(314)
					Granulocytopenia	A 67%; B 55%	
					Infection	A 21%; B 13%	
					Stomatitis	A 31%; B 0%	
Stage III or IV HNSCC	Phase III	IC (PF) followed by CRT (PF)	IC (PFD) followed by CRT (PFD)		Neutropenia	A 56%; B 83%	(315)
					Mucositis	A 27%; B 21%	
					Nausea and vomiting	A 24%; B 22%	
Unresectable HNSCC	Phase III	RT	CRT (P)	CRT (PF) split course	Mucositis/dysphagia	A 33%; B 45%; C 47%	(316)
					Leukopenia	A 1%; B 42%; C 31%	
					Anemia	A 0%; B 18%; C 19%	
High-Risk HNSCC	Phase III	RT after surgery	CRT (P) after surgery		Acute toxicity of mucous membrane	A 37%; B 62%	(317)
					Acute toxicity of pharynx/esophagus	A 32%; B 50%	
					Nausea and vomiting	A 0%; B 40%	
Advanced HNSCC	Phase III	RT	CRT (P)	IC (PF) followed by RT	Hematologic toxicity	A 3%; B 47%; C 67%	(318)
					Stomatitis	A 24%; B 43%; C 44%	
					Pharyngeal or esophageal toxicity	A 19%; B 35%; C 19%	

Abbreviations: **CT**, chemotherapy; **RT**, radiotherapy; **CRT**, concurrent chemoradiotherapy; **P**, cisplatin; **PF**, cisplatin and fluorouracil; **PT**, cisplatin and paclitaxel; **IC**, induction chemotherapy; **PFD**, cisplatin, fluorouracil, and docetaxel

6 FUTURE DIRECTIONS

A recent report from our laboratory has utilized a computational modeling to explore the direct molecular targets of garcinol and showed that garcinol could directly bind to the SH2 domain of STAT3 and suppress its dimerization *in vitro* (311). Elucidating the detailed molecular mechanism(s) by which garcinol can modulate multiple oncogenic signaling pathways will provide deeper understanding of its mechanisms of action against various tumor cells. Although garcinol has been reported to exhibit chemopreventive and tumor inhibitory effects in chemically-induced HNSCC models or subcutaneous xenografts, the efficacy of the drug in metastatic disease *in vivo* has not been studied yet. A well established cell line-derived or patient-derived tumor orthotopic xenograft model could be established to evaluate the anti-metastatic potential of garcinol *in vivo* against HNSCC. Diverse doses or concentrations of garcinol have been applied in various animal models, however, garcinol has not been so far tested in humans as an anticancer agent, and hence the clinically relevant doses are not clear as of yet. Thus, systematic study of the toxicology on garcinol should be addressed to obtain the safety profile of this natural agent.

REFERENCES

1. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature. 2001;411:342-8.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127:2893-917.
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011;61:69-90.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.
5. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57-70.
6. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature. 2001;411:375-9.
7. Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. The New England journal of medicine. 1993;328:184-94.
8. Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. Lancet. 2008;371:1695-709.
9. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA: a cancer journal for clinicians. 2010;60:277-300.
10. Chin D, Boyle GM, Porceddu S, Theile DR, Parsons PG, Coman WB. Head and neck cancer: past, present and future. Expert review of anticancer therapy. 2006;6:1111-8.
11. Maier H, Dietz A, Gewelke U, Heller WD, Weidauer H. Tobacco and alcohol and the risk of head and neck cancer. The Clinical investigator. 1992;70:320-7.
12. Hashibe M, Brennan P, Chuang SC, Boccia S, Castellsague X, Chen C, et al. Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2009;18:541-50.
13. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. Journal of the National Cancer Institute. 2000;92:709-20.

14. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. The lancet oncology. 2010;11:781-9.
15. Sankaranarayanan R, Masuyer E, Swaminathan R, Ferlay J, Whelan S. Head and neck cancer: a global perspective on epidemiology and prognosis. Anticancer research. 1998;18:4779-86.
16. Singapore Cancer Registry Interim Annual Registry Report. Trends in Cancer Incidence in Singapore, 2008-2012: National Registry of Diseases Office.
17. Head and Neck Cancer: Symptoms, Causes and Treatments. Available from: http://www.healthxchange.com.sg/healthyliving/SpecialFocus/Pages/Head-and-Neck-Cancer-Symptoms-Causes-and-Treatments.aspx
18. Pai SI, Westra WH. Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. Annual review of pathology. 2009;4:49-70.
19. Rosas SL, Koch W, da Costa Carvalho MG, Wu L, Califano J, Westra W, et al. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer research. 2001;61:939-42.
20. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer research. 2000;60:892-5.
21. Pfister DG, Ang KK, Brizel DM, Burtness BA, Cmelak AJ, Colevas AD, et al. Head and neck cancers. Journal of the National Comprehensive Cancer Network : JNCCN. 2011;9:596-650.
22. Patel SG, Shah JP. TNM staging of cancers of the head and neck: striving for uniformity among diversity. CA: a cancer journal for clinicians. 2005;55:242-58; quiz 61-2, 64.
23. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer. 1953;6:963-8.
24. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nature reviews Cancer. 2011;11:9-22.
25. Ha PK, Califano JA. The molecular biology of mucosal field cancerization of the head and neck. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. 2003;14:363-9.

26. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer research. 2003;63:1727-30.
27. Califano J, van der Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer research. 1996;56:2488-92.
28. Perez-Ordonez B, Beauchemin M, Jordan RC. Molecular biology of squamous cell carcinoma of the head and neck. J Clin Pathol. 2006;59:445-53.
29. Vogelstein B, Kinzler KW. The multistep nature of cancer. Trends in genetics : TIG. 1993;9:138-41.
30. Mao L, Lee JS, Fan YH, Ro JY, Batsakis JG, Lippman S, et al. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. Nature medicine. 1996;2:682-5.
31. van der Riet P, Nawroz H, Hruban RH, Corio R, Tokino K, Koch W, et al. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. Cancer research. 1994;54:1156-8.
32. Rocco JW, Sidransky D. p16(MTS-1/CDKN2/INK4a) in cancer progression. Experimental cell research. 2001;264:42-55.
33. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. Cell. 2007;130:223-33.
34. Callender T, el-Naggar AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG. PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. Cancer. 1994;74:152-8.
35. Pignataro L, Pruneri G, Carboni N, Capaccio P, Cesana BM, Neri A, et al. Clinical relevance of cyclin D1 protein overexpression in laryngeal squamous cell carcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1998;16:3069-77.
36. Capaccio P, Pruneri G, Carboni N, Pagliari AV, Quatela M, Cesana BM, et al. Cyclin D1 expression is predictive of occult metastases in head and neck cancer patients with clinically negative cervical lymph nodes. Head & neck. 2000;22:234-40.
37. Pande P, Mathur M, Shukla NK, Ralhan R. pRb and p16 protein alterations in human oral tumorigenesis. Oral oncology. 1998;34:396-403.
38. Xu J, Gimenez-Conti IB, Cunningham JE, Collet AM, Luna MA, Lanfranchi HE, et al. Alterations of p53, cyclin D1, Rb, and H-ras in human oral carcinomas related to tobacco use. Cancer. 1998;83:204-12.

39. El-Naggar AK, Lai S, Clayman GL, Zhou JH, Tucker SA, Myers J, et al. Expression of p16, Rb, and cyclin D1 gene products in oral and laryngeal squamous carcinoma: biological and clinical implications. Human pathology. 1999;30:1013-8.
40. Wiest T, Schwarz E, Enders C, Flechtenmacher C, Bosch FX. Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. Oncogene. 2002;21:1510-7.
41. Gonzalez MV, Pello MF, Lopez-Larrea C, Suarez C, Menendez MJ, Coto E. Loss of heterozygosity and mutation analysis of the p16 (9p21) and p53 (17p13) genes in squamous cell carcinoma of the head and neck. Clinical cancer research : an official journal of the American Association for Cancer Research. 1995;1:1043-9.
42. Balz V, Scheckenbach K, Gotte K, Bockmuhl U, Petersen I, Bier H. Is the p53 inactivation frequency in squamous cell carcinomas of the head and neck underestimated? Analysis of p53 exons 2-11 and human papillomavirus 16/18 E6 transcripts in 123 unselected tumor specimens. Cancer research. 2003;63:1188-91.
43. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. The New England journal of medicine. 2007;357:2552-61.
44. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990;63:1129-36.
45. Boyle JO, Hakim J, Koch W, van der Riet P, Hruban RH, Roa RA, et al. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer research. 1993;53:4477-80.
46. Herbst RS. Review of epidermal growth factor receptor biology. International journal of radiation oncology, biology, physics. 2004;59:21-6.
47. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Critical reviews in oncology/hematology. 1995;19:183-232.
48. Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. Molecular systems biology. 2005;1:2005 0010.

49. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. The EMBO journal. 2000;19:3159-67.
50. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Experimental cell research. 2003;284:31-53.
51. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. Nature reviews Cancer. 2004;4:361-70.
52. Scher HI, Sarkis A, Reuter V, Cohen D, Netto G, Petrylak D, et al. Changing pattern of expression of the epidermal growth factor receptor and transforming growth factor alpha in the progression of prostatic neoplasms. Clinical cancer research : an official journal of the American Association for Cancer Research. 1995;1:545-50.
53. Yamanaka Y, Friess H, Kobrin MS, Buchler M, Beger HG, Korc M. Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. Anticancer research. 1993;13:565-9.
54. Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF, Collins VP. Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. Cancer research. 1991;51:2164-72.
55. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. European journal of cancer. 2001;37 Suppl 4:S9-15.
56. Pedersen MW, Meltorn M, Damstrup L, Poulsen HS. The type III epidermal growth factor receptor mutation. Biological significance and potential target for anti-cancer therapy. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2001;12:745-60.
57. Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, et al. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer research. 1995;55:5536-9.
58. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366:2-16.
59. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer research. 1993;53:3579-84.

60. Ongkeko WM, Altuna X, Weisman RA, Wang-Rodriguez J. Expression of protein tyrosine kinases in head and neck squamous cell carcinomas. American journal of clinical pathology. 2005;124:71-6.
61. Rubin Grandis J, Melhem MF, Gooding WE, Day R, Holst VA, Wagener MM, et al. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. Journal of the National Cancer Institute. 1998;90:824-32.
62. Gupta AK, McKenna WG, Weber CN, Feldman MD, Goldsmith JD, Mick R, et al. Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8:885-92.
63. Quon H, Liu FF, Cummings BJ. Potential molecular prognostic markers in head and neck squamous cell carcinomas. Head & neck. 2001;23:147-59.
64. Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12:5064-73.
65. Rogers SJ, Harrington KJ, Rhys-Evans P, P OC, Eccles SA. Biological significance of c-erbB family oncogenes in head and neck cancer. Cancer metastasis reviews. 2005;24:47-69.
66. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell. 2002;109 Suppl:S121-31.
67. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264:1415-21.
68. Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. Nature reviews Molecular cell biology. 2002;3:651-62.
69. Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, et al. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. Cell. 1994;77:63-71.
70. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nature reviews Cancer. 2009;9:798-809.
71. Yu H, Jove R. The STATs of cancer--new molecular targets come of age. Nature reviews Cancer. 2004;4:97-105.

72. Hirano T, Ishihara K, Hibi M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene. 2000;19:2548-56.
73. Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, et al. STAT proteins: from normal control of cellular events to tumorigenesis. Journal of cellular physiology. 2003;197:157-68.
74. Yu CL, Meyer DJ, Campbell GS, Larner AC, Carter-Su C, Schwartz J, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science. 1995;269:81-3.
75. Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JE, Jr. Stat3 activation is required for cellular transformation by v-src. Molecular and cellular biology. 1998;18:2553-8.
76. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell. 1999;98:295-303.
77. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene. 2000;19:2474-88.
78. Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, Pitt AS, et al. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth In vitro. The Journal of clinical investigation. 1998;102:1385-92.
79. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity. 1999;10:105-15.
80. Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, et al. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. Oncogene. 2005;24:5552-60.
81. Xie TX, Huang FJ, Aldape KD, Kang SH, Liu M, Gershenwald JE, et al. Activation of stat3 in human melanoma promotes brain metastasis. Cancer research. 2006;66:3188-96.
82. Grandis JR, Drenning SD, Zeng Q, Watkins SC, Melhem MF, Endo S, et al. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2000;97:4227-32.
83. Song JI, Grandis JR. STAT signaling in head and neck cancer. Oncogene. 2000;19:2489-95.

84. Kijima T, Niwa H, Steinman RA, Drenning SD, Gooding WE, Wentzel AL, et al. STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth in vivo. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research. 2002;13:355-62.
85. Masuda M, Suzui M, Yasumatu R, Nakashima T, Kuratomi Y, Azuma K, et al. Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma. Cancer research. 2002;62:3351-5.
86. Shah NG, Trivedi TI, Tankshali RA, Goswami JV, Jetly DH, Shukla SN, et al. Prognostic significance of molecular markers in oral squamous cell carcinoma: a multivariate analysis. Head & neck. 2009;31:1544-56.
87. Leong PL, Andrews GA, Johnson DE, Dyer KF, Xi S, Mai JC, et al. Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proceedings of the National Academy of Sciences of the United States of America. 2003;100:4138-43.
88. Turkson J, Ryan D, Kim JS, Zhang Y, Chen Z, Haura E, et al. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. The Journal of biological chemistry. 2001;276:45443-55.
89. Masuda M, Suzui M, Weinstein IB. Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines. Clinical cancer research : an official journal of the American Association for Cancer Research. 2001;7:4220-9.
90. Leeman RJ, Lui VW, Grandis JR. STAT3 as a therapeutic target in head and neck cancer. Expert opinion on biological therapy. 2006;6:231-41.
91. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell. 1986;46:705-16.
92. Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annual review of immunology. 1996;14:649-83.
93. Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes & development. 2004;18:2195-224.

94. Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, et al. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science. 2001;293:1495-9.
95. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell. 2008;132:344-62.
96. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. Nature reviews Molecular cell biology. 2004;5:392-401.
97. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends in immunology. 2004;25:280-8.
98. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annual review of immunology. 1998;16:225-60.
99. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nature reviews Molecular cell biology. 2007;8:49-62.
100. Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-kappaB: its role in health and disease. Journal of molecular medicine. 2004;82:434-48.
101. Sarkar FH, Li Y, Wang Z, Kong D. NF-kappaB signaling pathway and its therapeutic implications in human diseases. International reviews of immunology. 2008;27:293-319.
102. Carrasco D, Rizzo CA, Dorfman K, Bravo R. The v-rel oncogene promotes malignant T-cell leukemia/lymphoma in transgenic mice. The EMBO journal. 1996;15:3640-50.
103. Gilmore TD. Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. Oncogene. 1999;18:6925-37.
104. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nature reviews Cancer. 2002;2:301-10.
105. Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13:1076-82.
106. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell. 2004;118:285-96.
107. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature. 2004;431:461-6.

108. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nature reviews Immunology. 2005;5:749-59.
109. Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. Current opinion in genetics & development. 2008;18:19-26.
110. Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nature immunology. 2002;3:221-7.
111. Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, et al. NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. Journal of immunology. 1994;153:2052-63.
112. Huang S, Robinson JB, Deguzman A, Bucana CD, Fidler IJ. Blockade of nuclear factor-kappaB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer research. 2000;60:5334-9.
113. Aggarwal BB. Nuclear factor-kappaB: the enemy within. Cancer cell. 2004;6:203-8.
114. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454:436-44.
115. Li F, Sethi G. Targeting transcription factor NF-kappaB to overcome chemoresistance and radioresistance in cancer therapy. Biochimica et biophysica acta. 2010;1805:167-80.
116. Tsurutani J, Castillo SS, Brognard J, Granville CA, Zhang C, Gills JJ, et al. Tobacco components stimulate Akt-dependent proliferation and NFkappaB-dependent survival in lung cancer cells. Carcinogenesis. 2005;26:1182-95.
117. Wolf JS, Chen Z, Dong G, Sunwoo JB, Bancroft CC, Capo DE, et al. IL (interleukin)-1alpha promotes nuclear factor-kappaB and AP-1-induced IL-8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2001;7:1812-20.
118. Bancroft CC, Chen Z, Yeh J, Sunwoo JB, Yeh NT, Jackson S, et al. Effects of pharmacologic antagonists of epidermal growth factor receptor, PI3K and MEK signal kinases on NF-kappaB and AP-1 activation and IL-8 and VEGF expression in human head and neck squamous cell carcinoma lines. Int J Cancer. 2002;99:538-48.
119. Mishra A, Bharti AC, Varghese P, Saluja D, Das BC. Differential expression and activation of NF-kappaB family proteins during oral

carcinogenesis: Role of high risk human papillomavirus infection. Int J Cancer. 2006;119:2840-50.
120. Nakayama H, Ikebe T, Beppu M, Shirasuna K. High expression levels of nuclear factor kappaB, IkappaB kinase alpha and Akt kinase in squamous cell carcinoma of the oral cavity. Cancer. 2001;92:3037-44.
121. Ondrey FG, Dong G, Sunwoo J, Chen Z, Wolf JS, Crowl-Bancroft CV, et al. Constitutive activation of transcription factors NF-(kappa)B, AP-1, and NF-IL6 in human head and neck squamous cell carcinoma cell lines that express pro-inflammatory and pro-angiogenic cytokines. Molecular carcinogenesis. 1999;26:119-29.
122. Bancroft CC, Chen Z, Dong G, Sunwoo JB, Yeh N, Park C, et al. Coexpression of proangiogenic factors IL-8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK-NF-kappaB signal pathways. Clinical cancer research : an official journal of the American Association for Cancer Research. 2001;7:435-42.
123. Loercher A, Lee TL, Ricker JL, Howard A, Geoghegen J, Chen Z, et al. Nuclear factor-kappaB is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. Cancer research. 2004;64:6511-23.
124. Allen CT, Ricker JL, Chen Z, Van Waes C. Role of activated nuclear factor-kappaB in the pathogenesis and therapy of squamous cell carcinoma of the head and neck. Head & neck. 2007;29:959-71.
125. Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS. Dysregulated molecular networks in head and neck carcinogenesis. Oral oncology. 2009;45:324-34.
126. Dong G, Loukinova E, Chen Z, Gangi L, Chanturita TI, Liu ET, et al. Molecular profiling of transformed and metastatic murine squamous carcinoma cells by differential display and cDNA microarray reveals altered expression of multiple genes related to growth, apoptosis, angiogenesis, and the NF-kappaB signal pathway. Cancer research. 2001;61:4797-808.
127. Chung CH, Parker JS, Ely K, Carter J, Yi Y, Murphy BA, et al. Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor-kappaB signaling as characteristics of a high-risk head and neck squamous cell carcinoma. Cancer research. 2006;66:8210-8.
128. Zhang PL, Pellitteri PK, Law A, Gilroy PA, Wood GC, Kennedy TL, et al. Overexpression of phosphorylated nuclear factor-kappa B in tonsillar squamous

cell carcinoma and high-grade dysplasia is associated with poor prognosis. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2005;18:924-32.
129. Duffey DC, Chen Z, Dong G, Ondrey FG, Wolf JS, Brown K, et al. Expression of a dominant-negative mutant inhibitor-kappaBalpha of nuclear factor-kappaB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth in vivo. Cancer research. 1999;59:3468-74.
130. Yu M, Yeh J, Van Waes C. Protein kinase casein kinase 2 mediates inhibitor-kappaB kinase and aberrant nuclear factor-kappaB activation by serum factor(s) in head and neck squamous carcinoma cells. Cancer research. 2006;66:6722-31.
131. Van Waes C, Chang AA, Lebowitz PF, Druzgal CH, Chen Z, Elsayed YA, et al. Inhibition of nuclear factor-kappaB and target genes during combined therapy with proteasome inhibitor bortezomib and reirradiation in patients with recurrent head-and-neck squamous cell carcinoma. International journal of radiation oncology, biology, physics. 2005;63:1400-12.
132. Wang F, Arun P, Friedman J, Chen Z, Van Waes C. Current and potential inflammation targeted therapies in head and neck cancer. Current opinion in pharmacology. 2009;9:389-95.
133. Vokes EE, Athanasiadis I. Chemotherapy of squamous cell carcinoma of head and neck: the future is now. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 1996;7:15-29.
134. Chen YJ, Chang JT, Liao CT, Wang HM, Yen TC, Chiu CC, et al. Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis. Cancer science. 2008;99:1507-14.
135. Fung C, Grandis JR. Emerging drugs to treat squamous cell carcinomas of the head and neck. Expert opinion on emerging drugs. 2010;15:355-73.
136. Dimery IW, Hong WK. Overview of combined modality therapies for head and neck cancer. Journal of the National Cancer Institute. 1993;85:95-111.
137. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. The New England journal of medicine. 2001;345:1890-900.
138. Mao L, Hong WK, Papadimitrakopoulou VA. Focus on head and neck cancer. Cancer cell. 2004;5:311-6.

139. Cohen EE, Lingen MW, Vokes EE. The expanding role of systemic therapy in head and neck cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2004;22:1743-52.
140. Kalyankrishna S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006;24:2666-72.
141. Chen LF, Cohen EE, Grandis JR. New strategies in head and neck cancer: understanding resistance to epidermal growth factor receptor inhibitors. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16:2489-95.
142. Sundvall M, Karrila A, Nordberg J, Grenman R, Elenius K. EGFR targeting drugs in the treatment of head and neck squamous cell carcinoma. Expert opinion on emerging drugs. 2010;15:185-201.
143. Seiwert TY, Cohen EE. Targeting angiogenesis in head and neck cancer. Seminars in oncology. 2008;35:274-85.
144. Pal SK, Reckamp K, Yu H, Figlin RA. Akt inhibitors in clinical development for the treatment of cancer. Expert opinion on investigational drugs. 2010;19:1355-66.
145. Aleshin A, Finn RS. SRC: a century of science brought to the clinic. Neoplasia. 2010;12:599-607.
146. Peng Z. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Human gene therapy. 2005;16:1016-27.
147. Bernier J. Head and neck cancer : multimodality management. New York: Springer; 2011.
148. Trotti A. Toxicity in head and neck cancer: a review of trends and issues. International journal of radiation oncology, biology, physics. 2000;47:1-12.
149. Widakowich C, de Castro G, Jr., de Azambuja E, Dinh P, Awada A. Review: side effects of approved molecular targeted therapies in solid cancers. The oncologist. 2007;12:1443-55.
150. Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. Cancer treatment reviews. 2007;33:9-23.
151. Haberman AB. Animal Models for Therapeutic Strategies Massachusetts: Cambridge Healthtech Institute; 2010.
152. Sano D, Myers JN. Xenograft models of head and neck cancers. Head & neck oncology. 2009;1:32.

153. Kawashiri S, Kumagai S, Kojima K, Harada H, Yamamoto E. Development of a new invasion and metastasis model of human oral squamous cell carcinomas. European journal of cancer Part B, Oral oncology. 1995;31B:216-21.
154. Myers JN, Holsinger FC, Jasser SA, Bekele BN, Fidler IJ. An orthotopic nude mouse model of oral tongue squamous cell carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8:293-8.
155. Qiu C, Wu H, He H, Qiu W. A cervical lymph node metastatic model of human tongue carcinoma: Serial and orthotopic transplantation of histologically intact patient specimens in nude mice. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2003;61:696-700.
156. Kim S, Park YW, Schiff BA, Doan DD, Yazici Y, Jasser SA, et al. An orthotopic model of anaplastic thyroid carcinoma in athymic nude mice. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005;11:1713-21.
157. Gelbard A, Kupferman ME, Jasser SA, Chen W, El-Naggar AK, Myers JN, et al. An orthotopic murine model of sinonasal malignancy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14:7348-57.
158. Younes MN, Park YW, Yazici YD, Gu M, Santillan AA, Nong X, et al. Concomitant inhibition of epidermal growth factor and vascular endothelial growth factor receptor tyrosine kinases reduces growth and metastasis of human salivary adenoid cystic carcinoma in an orthotopic nude mouse model. Molecular cancer therapeutics. 2006;5:2696-705.
159. Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. Nature reviews Drug discovery. 2006;5:741-54.
160. Prudencio J, Akutsu N, Benlimame N, Wang T, Bastien Y, Lin R, et al. Action of low calcemic 1alpha,25-dihydroxyvitamin D3 analogue EB1089 in head and neck squamous cell carcinoma. Journal of the National Cancer Institute. 2001;93:745-53.
161. Varadhachary A, Wolf JS, Petrak K, O'Malley BW, Jr., Spadaro M, Curcio C, et al. Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy. Int J Cancer. 2004;111:398-403.

162. Thomas GR, Chen Z, Enamorado I, Bancroft C, Van Waes C. IL-12- and IL-2-induced tumor regression in a new murine model of oral squamous-cell carcinoma is promoted by expression of the CD80 co-stimulatory molecule and interferon-gamma. Int J Cancer. 2000;86:368-74.
163. Khanna C, Hunter K. Modeling metastasis in vivo. Carcinogenesis. 2005;26:513-23.
164. Lu SL, Herrington H, Wang XJ. Mouse models for human head and neck squamous cell carcinomas. Head & neck. 2006;28:945-54.
165. Smith LP, Thomas GR. Animal models for the study of squamous cell carcinoma of the upper aerodigestive tract: a historical perspective with review of their utility and limitations. Part A. Chemically-induced de novo cancer, syngeneic animal models of HNSCC, animal models of transplanted xenogeneic human tumors. Int J Cancer. 2006;118:2111-22.
166. Lu SL, Reh D, Li AG, Woods J, Corless CL, Kulesz-Martin M, et al. Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. Cancer research. 2004;64:4405-10.
167. Lu SL, Herrington H, Reh D, Weber S, Bornstein S, Wang D, et al. Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. Genes & development. 2006;20:1331-42.
168. Caulin C, Nguyen T, Longley MA, Zhou Z, Wang XJ, Roop DR. Inducible activation of oncogenic K-ras results in tumor formation in the oral cavity. Cancer research. 2004;64:5054-8.
169. Corson TW, Crews CM. Molecular understanding and modern application of traditional medicines: triumphs and trials. Cell. 2007;130:769-74.
170. Efferth T, Li PC, Konkimalla VS, Kaina B. From traditional Chinese medicine to rational cancer therapy. Trends in molecular medicine. 2007;13:353-61.
171. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environmental health perspectives. 2001;109 Suppl 1:69-75.
172. Padhye S, Ahmad A, Oswal N, Sarkar FH. Emerging role of Garcinol, the antioxidant chalcone from Garcinia indica Choisy and its synthetic analogs. Journal of hematology & oncology. 2009;2:38.

173. Aggarwal BB, Kunnumakkara AB. Molecular targets and therapeutic uses of spices : modern uses for ancient medicine. Hackensack, NJ: World Scientific; 2009.
174. Yamaguchi F, Ariga T, Yoshimura Y, Nakazawa H. Antioxidative and anti-glycation activity of garcinol from Garcinia indica fruit rind. Journal of agricultural and food chemistry. 2000;48:180-5.
175. Yamaguchi F, Saito M, Ariga T, Yoshimura Y, Nakazawa H. Free radical scavenging activity and antiulcer activity of garcinol from Garcinia indica fruit rind. Journal of agricultural and food chemistry. 2000;48:2320-5.
176. Chatterjee A, Yasmin T, Bagchi D, Stohs SJ. The bactericidal effects of Lactobacillus acidophilus, garcinol and Protykin compared to clarithromycin, on Helicobacter pylori. Molecular and cellular biochemistry. 2003;243:29-35.
177. Liao CH, Sang S, Liang YC, Ho CT, Lin JK. Suppression of inducible nitric oxide synthase and cyclooxygenase-2 in downregulating nuclear factor-kappa B pathway by Garcinol. Molecular carcinogenesis. 2004;41:140-9.
178. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. BioEssays : news and reviews in molecular, cellular and developmental biology. 1998;20:615-26.
179. Roth SY, Denu JM, Allis CD. Histone acetyltransferases. Annual review of biochemistry. 2001;70:81-120.
180. Mahlknecht U, Hoelzer D. Histone acetylation modifiers in the pathogenesis of malignant disease. Molecular medicine. 2000;6:623-44.
181. Timmermann S, Lehrmann H, Polesskaya A, Harel-Bellan A. Histone acetylation and disease. Cellular and molecular life sciences : CMLS. 2001;58:728-36.
182. Sterner DE, Berger SL. Acetylation of histones and transcription-related factors. Microbiology and molecular biology reviews : MMBR. 2000;64:435-59.
183. Vo N, Goodman RH. CREB-binding protein and p300 in transcriptional regulation. The Journal of biological chemistry. 2001;276:13505-8.
184. Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. Genes & development. 2000;14:1553-77.
185. Iyer NG, Ozdag H, Caldas C. p300/CBP and cancer. Oncogene. 2004;23:4225-31.
186. Chan HM, La Thangue NB. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. Journal of cell science. 2001;114:2363-73.

187. Kouzarides T. Histone acetylases and deacetylases in cell proliferation. Current opinion in genetics & development. 1999;9:40-8.
188. Balasubramanyam K, Altaf M, Varier RA, Swaminathan V, Ravindran A, Sadhale PP, et al. Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. The Journal of biological chemistry. 2004;279:33716-26.
189. Collins HM, Abdelghany MK, Messmer M, Yue B, Deeves SE, Kindle KB, et al. Differential effects of garcinol and curcumin on histone and p53 modifications in tumour cells. BMC cancer. 2013;13:37.
190. Du T, Nagai Y, Xiao Y, Greene MI, Zhang H. Lysosome-dependent p300/FOXP3 degradation and limits Treg cell functions and enhances targeted therapy against cancers. Experimental and molecular pathology. 2013;95:38-45.
191. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860-7.
192. Tanaka T, Kohno H, Shimada R, Kagami S, Yamaguchi F, Kataoka S, et al. Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats. Carcinogenesis. 2000;21:1183-9.
193. Kiernan R, Bres V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, et al. Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. The Journal of biological chemistry. 2003;278:2758-66.
194. Batta K, Das C, Gadad S, Shandilya J, Kundu TK. Reversible acetylation of non histone proteins: role in cellular function and disease. Sub-cellular biochemistry. 2007;41:193-212.
195. Saha RN, Jana M, Pahan K. MAPK p38 regulates transcriptional activity of NF-kappaB in primary human astrocytes via acetylation of p65. Journal of immunology. 2007;179:7101-9.
196. Jing Y, Ai Q, Lin L, Dai J, Jia M, Zhou D, et al. Protective effects of garcinol in mice with lipopolysaccharide/D-galactosamine-induced apoptotic liver injury. International immunopharmacology. 2014;19:373-80.
197. Hsu CL, Lin YJ, Ho CT, Yen GC. The inhibitory effect of pterostilbene on inflammatory responses during the interaction of 3T3-L1 adipocytes and RAW 264.7 macrophages. Journal of agricultural and food chemistry. 2013;61:602-10.
198. Ahmad A, Wang Z, Ali R, Maitah MY, Kong D, Banerjee S, et al. Apoptosis-inducing effect of garcinol is mediated by NF-kappaB signaling in breast cancer cells. Journal of cellular biochemistry. 2010;109:1134-41.

199. Parasramka MA, Gupta SV. Garcinol inhibits cell proliferation and promotes apoptosis in pancreatic adenocarcinoma cells. Nutrition and cancer. 2011;63:456-65.
200. Ahmad A, Wang Z, Wojewoda C, Ali R, Kong D, Maitah MY, et al. Garcinol-induced apoptosis in prostate and pancreatic cancer cells is mediated by NF- kappaB signaling. Frontiers in bioscience. 2011;3:1483-92.
201. Ahmad A, Sarkar SH, Bitar B, Ali S, Aboukameel A, Sethi S, et al. Garcinol regulates EMT and Wnt signaling pathways in vitro and in vivo, leading to anticancer activity against breast cancer cells. Molecular cancer therapeutics. 2012;11:2193-201.
202. Hong J, Sang S, Park HJ, Kwon SJ, Suh N, Huang MT, et al. Modulation of arachidonic acid metabolism and nitric oxide synthesis by garcinol and its derivatives. Carcinogenesis. 2006;27:278-86.
203. Masullo M, Menegazzi M, Di Micco S, Beffy P, Bifulco G, Dal Bosco M, et al. Direct Interaction of Garcinol and Related Polyisoprenylated Benzophenones of Garcinia cambogia Fruits with the Transcription Factor STAT-1 as a Likely Mechanism of Their Inhibitory Effect on Cytokine Signaling Pathways. Journal of natural products. 2014.
204. Ahmad A, Sarkar SH, Aboukameel A, Ali S, Biersack B, Seibt S, et al. Anticancer action of garcinol in vitro and in vivo is in part mediated through inhibition of STAT-3 signaling. Carcinogenesis. 2012;33:2450-6.
205. Steele VE, Holmes CA, Hawk ET, Kopelovich L, Lubet RA, Crowell JA, et al. Lipoxygenase inhibitors as potential cancer chemopreventives. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 1999;8:467-83.
206. Wang D, Dubois RN. Prostaglandins and cancer. Gut. 2006;55:115-22.
207. Balsinde J, Balboa MA, Insel PA, Dennis EA. Regulation and inhibition of phospholipase A2. Annual review of pharmacology and toxicology. 1999;39:175-89.
208. Zha S, Yegnasubramanian V, Nelson WG, Isaacs WB, De Marzo AM. Cyclooxygenases in cancer: progress and perspective. Cancer letters. 2004;215:1-20.
209. Shureiqi I, Lippman SM. Lipoxygenase modulation to reverse carcinogenesis. Cancer research. 2001;61:6307-12.

210. Koeberle A, Northoff H, Werz O. Identification of 5-lipoxygenase and microsomal prostaglandin E2 synthase-1 as functional targets of the anti-inflammatory and anti-carcinogenic garcinol. Biochemical pharmacology. 2009;77:1513-21.
211. Chen X, Zhang X, Lu Y, Shim JY, Sang S, Sun Z, et al. Chemoprevention of 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster cheek pouch carcinogenesis by a 5-lipoxygenase inhibitor, garcinol. Nutrition and cancer. 2012;64:1211-8.
212. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiological reviews. 2001;81:807-69.
213. Ambrosino C, Nebreda AR. Cell cycle regulation by p38 MAP kinases. Biology of the cell / under the auspices of the European Cell Biology Organization. 2001;93:47-51.
214. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nature reviews Cancer. 2009;9:537-49.
215. Yu SY, Liao CH, Chien MH, Tsai TY, Lin JK, Weng MS. Induction of p21(Waf1/Cip1) by Garcinol via Downregulation of p38-MAPK Signaling in p53-Independent H1299 Lung Cancer. Journal of agricultural and food chemistry. 2014;62:2085-95.
216. McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. Nature reviews Cancer. 2005;5:505-15.
217. Liao CH, Sang S, Ho CT, Lin JK. Garcinol modulates tyrosine phosphorylation of FAK and subsequently induces apoptosis through down-regulation of Src, ERK, and Akt survival signaling in human colon cancer cells. Journal of cellular biochemistry. 2005;96:155-69.
218. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nature reviews Genetics. 2004;5:522-31.
219. Mattick JS, Makunin IV. Small regulatory RNAs in mammals. Human molecular genetics. 2005;14 Spec No 1:R121-32.
220. Tong AW, Nemunaitis J. Modulation of miRNA activity in human cancer: a new paradigm for cancer gene therapy? Cancer gene therapy. 2008;15:341-55.
221. Parasramka MA, Ali S, Banerjee S, Deryavoush T, Sarkar FH, Gupta S. Garcinol sensitizes human pancreatic adenocarcinoma cells to gemcitabine in

association with microRNA signatures. Molecular nutrition & food research. 2013;57:235-48.
222. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer science. 2007;98:1512-20.
223. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Current opinion in cell biology. 2005;17:548-58.
224. Egleton RD, Brown KC, Dasgupta P. Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis. Trends in pharmacological sciences. 2008;29:151-8.
225. Wessler I, Kirkpatrick CJ. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. British journal of pharmacology. 2008;154:1558-71.
226. Schuller HM. Is cancer triggered by altered signalling of nicotinic acetylcholine receptors? Nature reviews Cancer. 2009;9:195-205.
227. Chen CS, Lee CH, Hsieh CD, Ho CT, Pan MH, Huang CS, et al. Nicotine-induced human breast cancer cell proliferation attenuated by garcinol through down-regulation of the nicotinic receptor and cyclin D3 proteins. Breast cancer research and treatment. 2011;125:73-87.
228. Oike T, Ogiwara H, Torikai K, Nakano T, Yokota J, Kohno T. Garcinol, a histone acetyltransferase inhibitor, radiosensitizes cancer cells by inhibiting non-homologous end joining. International journal of radiation oncology, biology, physics. 2012;84:815-21.
229. Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis. 2000;21:485-95.
230. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell. 2002;108:153-64.
231. Prasad S, Ravindran J, Sung B, Pandey MK, Aggarwal BB. Garcinol potentiates TRAIL-induced apoptosis through modulation of death receptors and antiapoptotic proteins. Molecular cancer therapeutics. 2010;9:856-68.
232. Pan MH, Chang WL, Lin-Shiau SY, Ho CT, Lin JK. Induction of apoptosis by garcinol and curcumin through cytochrome c release and activation of caspases in human leukemia HL-60 cells. Journal of agricultural and food chemistry. 2001;49:1464-74.

233. Cheng AC, Tsai ML, Liu CM, Lee MF, Nagabhushanam K, Ho CT, et al. Garcinol inhibits cell growth in hepatocellular carcinoma Hep3B cells through induction of ROS-dependent apoptosis. Food & function. 2010;1:301-7.
234. Li F, Shanmugam MK, Chen L, Chatterjee S, Basha J, Kumar AP, et al. Garcinol, a polyisoprenylated benzophenone modulates multiple proinflammatory signaling cascades leading to the suppression of growth and survival of head and neck carcinoma. Cancer prevention research. 2013;6:843-54.
235. Dey A, Tergaonkar V, Lane DP. Double-edged swords as cancer therapeutics: simultaneously targeting p53 and NF-kappaB pathways. Nature reviews Drug discovery. 2008;7:1031-40.
236. Zimmermann GR, Lehar J, Keith CT. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug discovery today. 2007;12:34-42.
237. Parasramka MA, Gupta SV. Synergistic effect of garcinol and curcumin on antiproliferative and apoptotic activity in pancreatic cancer cells. Journal of oncology. 2012;2012:709739.
238. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene. 2003;22:8628-33.
239. Rowinsky EK. Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23:9394-407.
240. Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA repair. 2006;5:1042-8.
241. Bolderson E, Richard DJ, Zhou BB, Khanna KK. Recent advances in cancer therapy targeting proteins involved in DNA double-strand break repair. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15:6314-20.
242. Oike T, Ogiwara H, Amornwichet N, Nakano T, Kohno T. Chromatin-regulating proteins as targets for cancer therapy. Journal of radiation research. 2014.
243. Lippman SM, Benner SE, Hong WK. Cancer chemoprevention. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1994;12:851-73.
244. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nature reviews Cancer. 2003;3:768-80.

245. Yoshida K, Tanaka T, Hirose Y, Yamaguchi F, Kohno H, Toida M, et al. Dietary garcinol inhibits 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats. Cancer letters. 2005;221:29-39.
246. Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. Cellular and molecular life sciences : CMLS. 2005;62:971-88.
247. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of immunological methods. 1995;184:39-51.
248. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. Journal of immunological methods. 1991;139:271-9.
249. Allen RT, Hunter WJ, 3rd, Agrawal DK. Morphological and biochemical characterization and analysis of apoptosis. Journal of pharmacological and toxicological methods. 1997;37:215-28.
250. Cohen GM. Caspases: the executioners of apoptosis. The Biochemical journal. 1997;326 (Pt 1):1-16.
251. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene. 2006;25:4798-811.
252. Tsuruo T, Naito M, Tomida A, Fujita N, Mashima T, Sakamoto H, et al. Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. Cancer science. 2003;94:15-21.
253. Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, et al. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 1999;5:1369-79.
254. Ranieri G, Patruno R, Ruggieri E, Montemurro S, Valerio P, Ribatti D. Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: from the biology to the clinic. Current medicinal chemistry. 2006;13:1845-57.
255. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nature reviews Cancer. 2002;2:563-72.
256. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nature reviews Cancer. 2003;3:362-74.
257. Schindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. The Journal of biological chemistry. 2007;282:20059-63.

258. Xu D, Qu CK. Protein tyrosine phosphatases in the JAK/STAT pathway. Frontiers in bioscience : a journal and virtual library. 2008;13:4925-32.
259. Knowles JA, Golden B, Yan L, Carroll WR, Helman EE, Rosenthal EL. Disruption of the AKT pathway inhibits metastasis in an orthotopic model of head and neck squamous cell carcinoma. The Laryngoscope. 2011;121:2359-65.
260. Hildebrandt MA, Lippman SM, Etzel CJ, Kim E, Lee JJ, Khuri FR, et al. Genetic variants in the PI3K/PTEN/AKT/mTOR pathway predict head and neck cancer patient second primary tumor/recurrence risk and response to retinoid chemoprevention. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18:3705-13.
261. Subramaniam A, Shanmugam MK, Perumal E, Li F, Nachiyappan A, Dai X, et al. Potential role of signal transducer and activator of transcription (STAT)3 signaling pathway in inflammation, survival, proliferation and invasion of hepatocellular carcinoma. Biochimica et biophysica acta. 2013;1835:46-60.
262. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. Journal of cellular physiology. 2000;182:311-22.
263. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer research. 2004;64:7099-109.
264. Chuang SE, Yeh PY, Lu YS, Lai GM, Liao CM, Gao M, et al. Basal levels and patterns of anticancer drug-induced activation of nuclear factor-kappaB (NF-kappaB), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells. Biochemical pharmacology. 2002;63:1709-16.
265. Almeida LO, Abrahao AC, Rosselli-Murai LK, Giudice FS, Zagni C, Leopoldino AM, et al. NFkappaB mediates cisplatin resistance through histone modifications in head and neck squamous cell carcinoma (HNSCC). FEBS open bio. 2014;4:96-104.
266. Browman GP, Hodson DI, Mackenzie RJ, Bestic N, Zuraw L, Cancer Care Ontario Practice Guideline Initiative H, et al. Choosing a concomitant chemotherapy and radiotherapy regimen for squamous cell head and neck cancer: A systematic review of the published literature with subgroup analysis. Head & neck. 2001;23:579-89.
267. Seiwert TY, Salama JK, Vokes EE. The chemoradiation paradigm in head and neck cancer. Nature clinical practice Oncology. 2007;4:156-71.

268. Cragg GM, Grothaus PG, Newman DJ. Impact of natural products on developing new anti-cancer agents. Chemical reviews. 2009;109:3012-43.
269. Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. Cancer. 1994;73:2013-26.
270. Hong J, Kwon SJ, Sang S, Ju J, Zhou JN, Ho CT, et al. Effects of garcinol and its derivatives on intestinal cell growth: Inhibitory effects and autoxidation-dependent growth-stimulatory effects. Free radical biology & medicine. 2007;42:1211-21.
271. Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2008;105:2415-20.
272. Limsirichaikul S, Niimi A, Fawcett H, Lehmann A, Yamashita S, Ogi T. A rapid non-radioactive technique for measurement of repair synthesis in primary human fibroblasts by incorporation of ethynyl deoxyuridine (EdU). Nucleic acids research. 2009;37:e31.
273. Sherr CJ. The Pezcoller lecture: cancer cell cycles revisited. Cancer research. 2000;60:3689-95.
274. Sherr CJ. Cancer cell cycles. Science. 1996;274:1672-7.
275. Deshpande A, Sicinski P, Hinds PW. Cyclins and cdks in development and cancer: a perspective. Oncogene. 2005;24:2909-15.
276. Bartkova J, Lukas J, Muller H, Strauss M, Gusterson B, Bartek J. Abnormal patterns of D-type cyclin expression and G1 regulation in human head and neck cancer. Cancer research. 1995;55:949-56.
277. Porter PL, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, et al. Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nature medicine. 1997;3:222-5.
278. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998;281:1322-6.
279. Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998;281:1309-12.
280. Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. Genes & development. 1999;13:239-52.
281. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer research. 1998;58:5315-20.

282. Scaffidi C, Schmitz I, Krammer PH, Peter ME. The role of c-FLIP in modulation of CD95-induced apoptosis. The Journal of biological chemistry. 1999;274:1541-8.
283. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000;407:249-57.
284. Ninck S, Reisser C, Dyckhoff G, Helmke B, Bauer H, Herold-Mende C. Expression profiles of angiogenic growth factors in squamous cell carcinomas of the head and neck. Int J Cancer. 2003;106:34-44.
285. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nature reviews Cancer. 2002;2:161-74.
286. Tang DG, Honn KV. Adhesion molecules and tumor metastasis: an update. Invasion & metastasis. 1994;14:109-22.
287. Kobayashi H, Boelte KC, Lin PC. Endothelial cell adhesion molecules and cancer progression. Current medicinal chemistry. 2007;14:377-86.
288. Uchida D, Begum NM, Almofti A, Nakashiro K, Kawamata H, Tateishi Y, et al. Possible role of stromal-cell-derived factor-1/CXCR4 signaling on lymph node metastasis of oral squamous cell carcinoma. Experimental cell research. 2003;290:289-302.
289. Muller A, Sonkoly E, Eulert C, Gerber PA, Kubitza R, Schirlau K, et al. Chemokine receptors in head and neck cancer: association with metastatic spread and regulation during chemotherapy. Int J Cancer. 2006;118:2147-57.
290. Tan CT, Chu CY, Lu YC, Chang CC, Lin BR, Wu HH, et al. CXCL12/CXCR4 promotes laryngeal and hypopharyngeal squamous cell carcinoma metastasis through MMP-13-dependent invasion via the ERK1/2/AP-1 pathway. Carcinogenesis. 2008;29:1519-27.
291. Yoon Y, Liang Z, Zhang X, Choe M, Zhu A, Cho HT, et al. CXC chemokine receptor-4 antagonist blocks both growth of primary tumor and metastasis of head and neck cancer in xenograft mouse models. Cancer research. 2007;67:7518-24.
292. Samara GJ, Lawrence DM, Chiarelli CJ, Valentino MD, Lyubsky S, Zucker S, et al. CXCR4-mediated adhesion and MMP-9 secretion in head and neck squamous cell carcinoma. Cancer letters. 2004;214:231-41.
293. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8:945-54.

294. Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, et al. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene. 2001;20:2499-513.
295. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. The Biochemical journal. 2003;374:1-20.
296. Pathak AK, Bhutani M, Nair AS, Ahn KS, Chakraborty A, Kadara H, et al. Ursolic acid inhibits STAT3 activation pathway leading to suppression of proliferation and chemosensitization of human multiple myeloma cells. Molecular cancer research : MCR. 2007;5:943-55.
297. Kim HY, Park EJ, Joe EH, Jou I. Curcumin suppresses Janus kinase-STAT inflammatory signaling through activation of Src homology 2 domain-containing tyrosine phosphatase 2 in brain microglia. Journal of immunology. 2003;171:6072-9.
298. Chen KF, Tai WT, Liu TH, Huang HP, Lin YC, Shiau CW, et al. Sorafenib overcomes TRAIL resistance of hepatocellular carcinoma cells through the inhibition of STAT3. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16:5189-99.
299. Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends in biochemical sciences. 2005;30:43-52.
300. Sethi G, Ahn KS, Sandur SK, Lin X, Chaturvedi MM, Aggarwal BB. Indirubin enhances tumor necrosis factor-induced apoptosis through modulation of nuclear factor-kappa B signaling pathway. The Journal of biological chemistry. 2006;281:23425-35.
301. Jackson-Bernitsas DG, Ichikawa H, Takada Y, Myers JN, Lin XL, Darnay BG, et al. Evidence that TNF-TNFR1-TRADD-TRAF2-RIP-TAK1-IKK pathway mediates constitutive NF-kappaB activation and proliferation in human head and neck squamous cell carcinoma. Oncogene. 2007;26:1385-97.
302. Konstantinopoulos PA, Fountzilas E, Pillay K, Zerbini LF, Libermann TA, Cannistra SA, et al. Carboplatin-induced gene expression changes in vitro are prognostic of survival in epithelial ovarian cancer. BMC medical genomics. 2008;1:59.
303. Lee TK, Poon RT, Wo JY, Ma S, Guan XY, Myers JN, et al. Lupeol suppresses cisplatin-induced nuclear factor-kappaB activation in head and neck

squamous cell carcinoma and inhibits local invasion and nodal metastasis in an orthotopic nude mouse model. Cancer research. 2007;67:8800-9.
304. Stewart DJ. Mechanisms of resistance to cisplatin and carboplatin. Critical reviews in oncology/hematology. 2007;63:12-31.
305. Duarte VM, Han E, Veena MS, Salvado A, Suh JD, Liang LJ, et al. Curcumin enhances the effect of cisplatin in suppression of head and neck squamous cell carcinoma via inhibition of IKKbeta protein of the NFkappaB pathway. Molecular cancer therapeutics. 2010;9:2665-75.
306. Lee H, Herrmann A, Deng JH, Kujawski M, Niu G, Li Z, et al. Persistently activated Stat3 maintains constitutive NF-kappaB activity in tumors. Cancer cell. 2009;15:283-93.
307. Squarize CH, Castilho RM, Sriuranpong V, Pinto DS, Jr., Gutkind JS. Molecular cross-talk between the NFkappaB and STAT3 signaling pathways in head and neck squamous cell carcinoma. Neoplasia. 2006;8:733-46.
308. Bollrath J, Greten FR. IKK/NF-kappaB and STAT3 pathways: central signalling hubs in inflammation-mediated tumour promotion and metastasis. EMBO reports. 2009;10:1314-9.
309. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine & growth factor reviews. 2010;21:11-9.
310. Sethi G, Ahn KS, Sung B, Kunnumakkara AB, Chaturvedi MM, Aggarwal BB. SH-5, an AKT inhibitor potentiates apoptosis and inhibits invasion through the suppression of anti-apoptotic, proliferative and metastatic gene products regulated by IkappaBalpha kinase activation. Biochemical pharmacology. 2008;76:1404-16.
311. Sethi G, Chatterjee S, Rajendran P, Li F, Shanmugam MK, Wong KF, et al. Inhibition of STAT3 dimerization and acetylation by garcinol suppresses the growth of human hepatocellular carcinoma in vitro and in vivo. Molecular cancer. 2014;13:66.
312. Carroll RE, Benya RV, Turgeon DK, Vareed S, Neuman M, Rodriguez L, et al. Phase IIa Clinical Trial of Curcumin for the Prevention of Colorectal Neoplasia. Cancer prevention research. 2011;4:354-64.
313. Dhillon N, Aggarwal BB, Newman RA, Wolf RA, Kunnumakkara AB, Abbruzzese JL, et al. Phase II trial of curcumin in patients with advanced pancreatic cancer. Clinical Cancer Research. 2008;14:4491-9.

314. Lazarevic B, Boezelijn G, Diep LM, Kvernrod K, Ogren O, Ramberg H, et al. Efficacy and Safety of Short-Term Genistein Intervention in Patients with Localized Prostate Cancer Prior to Radical Prostatectomy: A Randomized, Placebo-Controlled, Double-Blind Phase 2 Clinical Trial. Nutrition and Cancer-an International Journal. 2011;63:889-98.
315. Miyanaga N, Akaza H, Hinotsu S, Fujioka T, Naito S, Namiki M, et al. Prostate Cancer Chemoprevention Study: An investigative randomized control study using purified isoflavones in men with rising prostate-specific antigen. Cancer science. 2012;103:125-30.
316. Lin TS, Ruppert AS, Johnson AJ, Fischer B, Heerema NA, Andritsos LA, et al. Phase II Study of Flavopiridol in Relapsed Chronic Lymphocytic Leukemia Demonstrating High Response Rates in Genetically High-Risk Disease. Journal of Clinical Oncology. 2009;27:6012-8.
317. Karp JE, Garrett-Mayer E, Estey EH, Rudek MA, Smith BD, Greer JM, et al. Randomized phase II study of two schedules of flavopiridol given as timed sequential therapy with cytosine arabinoside and mitoxantrone for adults with newly diagnosed, poor-risk acute myelogenous leukemia. Haematol-Hematol J. 2012;97:1736-42.
318. Tsao AS, Liu D, Martin J, Tang XM, Lee JJ, El-Naggar AK, et al. Phase II Randomized, Placebo-Controlled Trial of Green Tea Extract in Patients with High-Risk Oral Premalignant Lesions. Cancer prevention research. 2009;2:931-41.
319. Shanafelt TD, Call TG, Zent CS, Leis JF, LaPlant B, Bowen DA, et al. Phase 2 trial of daily, oral polyphenon E in patients with asymptomatic, Rai stage 0 to II chronic lymphocytic leukemia. Cancer. 2013;119:363-70.
320. Ang KK, Harris J, Garden AS, Trotti A, Jones CU, Carrascosa L, et al. Concomitant boost radiation plus concurrent cisplatin for advanced head and neck carcinomas: Radiation therapy oncology group phase II trial 99-14. Journal of Clinical Oncology. 2005;23:3008-15.
321. Vokes EE, Kies MS, Haraf DJ, Stenson K, List M, Humerickhouse R, et al. Concomitant chemoradiotherapy as primary therapy for locoregionally advanced head and neck cancer. Journal of Clinical Oncology. 2000;18:1652-61.
322. Gibson MK, Li Y, Murphy B, Hussain MH, DeConti RC, Ensley J, et al. Randomized phase III evaluation of cisplatin plus fluorouracil versus cisplatin plus paclitaxel in advanced head and neck cancer (E1395): an intergroup trial of the Eastern Cooperative Oncology Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23:3562-7.

323. Posner MR, Hershock DM, Blajman CR, Mickiewicz E, Winquist E, Gorbounova V, et al. Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer. New Engl J Med. 2007;357:1705-15.
324. Adelstein DJ, Li Y, Adams GL, Wagner H, Jr., Kish JA, Ensley JF, et al. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21:92-8.
325. Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. The New England journal of medicine. 2004;350:1937-44.
326. Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. New Engl J Med. 2003;349:2091-8.